Impact of Nuclear EDS1
on Arabidopsis Immunity

Inaugural-Dissertation
zur
Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Universität zu Köln

vorgelegt von

Nora Anna Peine

aus Krefeld

Köln 2013

Diese Arbeit wurde durchgeführt am Max-Planck-Institut für Pflanzenzüchtungsforschung in Köln in der Abteilung für Molekulare Phytopathologie (Direktor: Prof. Dr. P. Schulze-Lefert).

MAX-PLANCK-GESELLSCHAFT

Max-Planck-Institut für
Pflanzenzüchtungsforschung

Berichterstatter:	Prof. Dr. Paul Schulze-Lefert Prof. Dr. Ute Höcker Dr. Laurent Deslandes
Prüfungsvorsitzender:	Prof. Dr. Martin Hülskamp
Tag der Disputation:	19.04.2013

Publications

- **Kim TH, Hauser F, Ha T, Xue S, Böhmer M, Nishimura N, Munemasa S, Hubbard K, Peine N, Lee BH, Lee S, Robert N, Parker JE, Schroeder JI** (2011). Chemical genetics reveals negative regulation of abscisic acid signaling by a plant immune response pathway. Current Biology; Volume 21, Issue 11, Pages 990-7

Contents

Abbreviations

-	fused to (in the context of gene/protein fusion constructs)
°C	degree Celsius
Avr	avirulence
bp	base pair(s)
CC	coiled-coil
cDNA	complementary DNA
cfu	colony forming unit
CLSM	confocal laser scanning microscopy
d	day(s)
dH2O	deionised water
ddH2O	deionised distilled water
Dex	dexamethasone
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleosidetriphosphate
dpi	day(s) post infection
EDS1	Enhanced Disease Susceptibility1
EDTA	ethylenediaminetetraacetic acid
ETI	effector-triggered immunity
EtOH	ethanol
FW	fresh weight
g	gram(s)
gDNA	genomic DNA
GR	glucocorticoid receptor hormone binding domain
h	hour(s)
Hpa	*Hyaloperonospora arabidopsidis*
hpi	hour(s) post infection/induction
HR	hypersensitive response
ICS1	Isochorismate Synthase1
kb	kilobase(s)
kDa	kiloDalton(s)
l	litre
LRR	leucine-rich repeats

M	molar (mol/l)
m	milli
μ	micro
MAMP	microbe-associated molecular patterns
MAPK	mitogen-activated protein kinase
min	minute(s)
mM	millimolar
mRNA	messenger ribonucleic acid
MW	molecular weight
mYFP	monomeric YFP
NB	nucleotide binding site
NES	nuclear export signal
ng	nanogram
NLR	NOD-like receptor
nls	non-functional nuclear localization signal
NLS	nuclear localization signal
nm	nanometer
NOD	nucleotide-binding oligomerization domain
NPC	nuclear pore complex
NPR1	Nonexpresser of PR genes1
N-terminal	amino-terminal
Nup	nucleoporin
OD	optical density
p35S	35S promoter of CaMV
PAA	polyacrylamide
PAD4	Phytoalexin Deficient4
PAGE	polyacrylamide gel-electrophoresis
PCR	polymerase chain reaction
pEDS1	native *EDS1* promoter
PEPC	phosphoenolpyruvate carboxylase
pH	negative decimal logarithm of the H+ concentration
PR	pathogenesis related
PRR	PAMP/pattern recognition receptor
Pst	*Pseudomonas syringae* pv. *tomato*
PTI	PAMP-triggered immunity
pv.	pathovar
R	resistance
RNA	ribonucleic acid
rpm	rounds per minute
RPM	resistance to *Pseudomonas syringae* pv. *maculicola*
RPP	resistance to *Peronospora parasitica*

RPS	resistance to *Pseudomonas syringae*
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SA	salicylic acid
SAG101	Senescence Associated Gene101
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
sec	second(s)
SID2	Salicylic Acid Induction Deficient2
TBS	Tris buffered saline
TIR	Drosophila Toll and mammalian interleukin-1 receptor
Tris	Tris-(hydroxymethyl)-aminomethane
U	unit
V	Volt(s)
v/v	volume per volume
w/v	weight per volume
wt	wild-type
YFP	yellow fluorescent protein

Summary

In nature, plants are constantly exposed to microbial pathogens and have evolved an effective and dynamic immune system in order to survive. Arabidopsis EDS1 (ENHANCED DISEASE SUSCEPTIBILITY1), with its interacting partners PAD4 (PHYTOALEXIN DEFICIENT4) and SAG101 (SENESCENCE ASSOCIATED GENE101), forms an important disease resistance signaling hub. EDS1 is essential for basal defense against biotrophic and hemibiotrophic pathogens and for effector-triggered resistance mediated by TIR (Toll-Interleukin1 receptor)-NB-LRR (Nucleotide Binding Site-Leucine Rich Repeat) receptors. Previous work suggested different functions of cytoplasmic and nuclear EDS1 pools in molecularly and spatially distinct complexes with its partners PAD4 and SAG101 and other resistance components. To gain deeper insights into the functional significance of EDS1 intracellular distribution, I investigated transgenic Arabidopsis plants that have enforced EDS1 nuclear accumulation through EDS1 fusion to a nuclear localization signal (EDS1-NLS).

My work shows that nuclear EDS1 is sufficient to confer basal and TIR-NB-LRR-triggered resistance to several tested pathogens. Furthermore, plants with nuclear-restricted EDS1 are able to signal TIR-NB-LRR-conditioned cell death. Whereas plants expressing moderate amounts of enforced nuclear EDS1 display wt-like defense, high levels of nuclear-restricted EDS1 induce defense outputs, such as defense-related transcriptional reprogramming and elevated levels of the phytohormone salicylic acid, without a pathogen stimulus. The amplitude of defense activation correlates with the severity of growth defects in EDS1-NLS plants. A certain threshold of nuclear EDS1 accumulation has to be passed before auto-activation of defense outputs is induced. Despite activated defenses, these plants exhibit only marginally enhanced basal resistance. The EDS1-NLS-conditioned defense phenotypes require *PAD4*, but not *SAG101*, and are largely independent of the SA defense signaling pathway, thus emphasizing the importance of a previously identified but poorly defined SA-independent branch of EDS1/PAD4 signaling. Notably, the presence of wild type EDS1 combined with high levels of nuclear-restricted EDS1 abolishes the auto-induced defense outputs, suggesting a counter-balancing role of cytosolic EDS1 and/or nucleo-cytoplasmic shuttling in modulating nuclear EDS1 activities. Experiments were initiated to distinguish prolonged and potentially pleiotropic effects of EDS1 nuclear restriction from its immediate consequences on resistance and development. Analysis of *in vivo* EDS1 nuclear interactors by co-immunoprecipitation and tandem mass spectrometry revealed an association of EDS1 with RPN2 which is part of the 26S proteasome, indicating a possible role of EDS1 in proteasome-mediated regulation of resistance responses. Taken together, the results of this study emphasize a crucial function of nuclear EDS1 in regulating plant immunity.

Zusammenfassung

Pflanzen besitzen ein effizientes und dynamisches Abwehrsystem, das sie gegenüber einem breiten Spektrum von Krankheitserregern schützt. In Arabidopsis stellt EDS1 (ENHANCED DISEASE SUSCEPTIBILITY1) mit seinen Interaktionspartnern PAD4 (PHYTOALEXIN DEFICIENT4) und SAG101 (SENESCENCE ASSOCIATED GENE101) einen wichtigen regulatorischen Knotenpunkt in der Pathogenabwehr dar. EDS1 ist essentiell sowohl in der basale Abwehr von biotrophen und hemibiotrophen Pathogenen, als auch in der Resistenz die von TIR-NB-LRR Rezeptoren vermittelt wird. Vorangegangene Arbeiten deuten auf verschiedene Funktionen von EDS1 im Zytoplasma und im Zellkern hin; diese beruhen vermutlich auf den räumlich getrennten und molekular unterschiedlichen Komplexen von EDS1 mit seinen Interaktoren PAD4, SAG101 oder möglichen weiteren Komponenten des Immunsystems. Um tiefere Einblicke in die funktionelle Bedeutung der EDS1-Verteilung innerhalb der Zelle zu erhalten wurden in der vorliegenden Arbeit transgene Arabidopsis-Linien untersucht, in denen EDS1 durch das Anhängen eines nukleären Lokalisationssignals in den Zellkern gezwungen wird (EDS1-NLS).

Meine Arbeit zeigt, dass auf den Zellkern begrenztes EDS1 hinreichend ist zur Abwehr der untersuchten Pathogene. Zusätzlich sind Pflanzen mit nukleär-begrenztem EDS1 in der Lage, Zelltod nach Pathogenerkennung durch Rezeptorproteine auszulösen. Pflanzen mit gemäßigtem, vorwiegend nukleärem EDS1-Gehalt weisen eine wildtyp-ähnliche Immunantwort auf. Pflanzen mit hoher Expression von nukleär lokalisiertem EDS1 hingegen zeigen induzierte Abwehrreaktionen, gekennzeichnet durch veränderte Genexpression und erhöhte Konzentration des Phytohormons Salicylsäure. Die Stärke der auto-induzierten Abwehraktivierung korreliert mit der Ausprägung morphologischer Veränderungen der EDS1-NLS Linien. Zudem muss sich eine bestimmte Menge an nukleärem EDS1 angesammelt haben, bevor Abwehrreaktionen ausgelöst werden. Trotz aktivierter Immunantwort weisen die Pflanzen nur geringfügig erhöhte basale Resistenz auf. Die induzierten Abwehrprozesse in EDS1-NLS Pflanzenlinien benötigen funktionales PAD4, sind aber unabhängig von SAG101 sowie von Komponenten des Salicylsäure-Signalwegs. Dies unterstreicht die Bedeutung eines Salicylsäure -unabhängigen Signalwegs in EDS1-vermittelter Resistenz, welcher schon in vorherigen Arbeiten beschrieben wurde, aber bis heute nicht umfassend verstanden ist. Expression von nukleär-zytoplasmatisch lokalisierten Wildtyp-EDS1 zusätzlich zu hohen Anteilen von nukleärem EDS1 führt zur Unterdrückung der auto-induzierten Immunantworten. Dies deutet darauf hin, dass zytoplasmatisches EDS1 der Aktivität von nukleärem EDS1 entgegenwirkt oder der Transport von EDS1 zwischen den Zellkompartimenten für die Regulation nukleärer EDS1-Aktivität von Bedeutung ist. Um zwischen den

Auswirkungen von dauerhaften, womöglich für die Pflanze schädlichen, und kurzzeitigen Effekten nukleärer EDS1-Aktivität zu unterscheiden, wurden bereits weiterführende Experimente initiiert. Massenspektrometrie-Analysen zur Identifikation von EDS1-assoziierten Proteinen im Zellkern ergaben RPN2 als möglichen Interaktionspartner. RPN2 ist Teil des 26S-Proteasoms. Dies lässt auf eine mögliche Beteiligung von EDS1 in der Regulierung von Abwehrprozessen durch den Proteasomkomplex schließen. Die Ergebnisse dieser Arbeit weisen auf eine Schlüsselrolle von nukleärem EDS1 in der Regulation des pflanzlichen Immunsystems hin.

1 Introduction

1.1 Plant immunity

Throughout their life, plants are constantly exposed to pathogens. A first barrier of protection against non-specialized pathogens is provided by waxy cuticles on the plant surface to resist pathogen penetration and by preformed anti-microbial compounds. During evolution, plants developed an effective inducible defense system to counteract harmful infections by microbes (Jones and Dangl, 2006). Like other organisms, plants have the ability to recognize pathogens and activate a broad defense program upon recognition. Plant immunity is commonly classified into two layers depending on the mode of pathogen perception (Jones and Dangl, 2006).

1.1.1 First layer – recognition of conserved microbial patterns

On the cell surface, plants express pattern recognition receptors (PRR). These receptors perceive highly conserved molecular signatures of microbes, referred to as microbe-associated molecular patterns (MAMPs). Interaction of PRRs with their corresponding MAMPs initiates a number of defense responses, such as the induction of MAP (mitogen activated protein) kinase signaling, production of reactive oxygen species, callose deposition at the site of infection and transcriptional activation of defense-related genes. Activation of these immune responses contributes to arrest pathogen growth and is called MAMP-triggered immunity (MTI) (Jones and Dangl, 2006). So far characterized PRRs in plants belong to the family of receptor-like kinases (RLKs). The best-analyzed plant responses to MAMPs are upon recognition of bacterial flagellin and bacterial elongation factor Tu by the receptor-like kinases FLS2 (FLAGELLIN SENSING 2) and EFR (EF-TU RECEPTOR), respectively (Gómez-Gómez and Boller, 2002; Zipfel et al., 2006) and perception of fungal chitin oligosaccharides by the LysM-RLK CERK1 (CHITIN ELICITOR RECEPTOR KINASE 1) receptor (Miya et al., 2007). To dampen MTI for a successful invasion of the plant, pathogens deliver effector proteins into the plant cell. Effectors can interfere with MTI at different stages during infection e.g. at the level of perception or by disrupting defense signaling, thereby enabling colonization of the host. In this case, the pathogen is denoted as virulent and the plant-pathogen interaction is classified as compatible (Chisholm et al., 2006). The residual level of immunity displayed by plants is referred to as basal resistance (Jones and Dangl, 2006).

1.1.2 Second layer – NB-LRR receptor mediated resistance

In contrast to animals, plants lack an adaptive immune system based on mobile defender cells and somatic adaptive antibodies (Ausubel, 2005). They evolved an alternative strategy to cope with host-adapted pathogens by sensing pathogen-derived effectors via cultivar specific resistance (*R*) genes in each cell (Dangl and Jones, 2001). R proteins are composed of a central nucleotide binding (NB)- and a C-terminal leucine-rich repeat (LRR)-domain. Plant R proteins are structurally related to animal NLR proteins (nucleotide-binding oligomerization domain (NOD)- and LRR-containing proteins). However, evidence points to a convergent evolution of the plant and animal immune system (Ausubel, 2005).

R proteins can be divided into two main subclasses depending on their N-terminal domain (Meyers et al., 2003). The N-terminus of one subclass resembles the *Drosophila* and mammalian Toll-Interleukin1 Receptor (TIR), whereas the other class possesses a N-terminal coiled-coil (CC) domain. Around 150 *R* genes were identified in the genome of *Arabidopsis thaliana* (hereafter referred to as Arabidopsis) and more than 600 in rice (*Oryza sativa*) (Meyers et al., 2003; Goff et al., 2002). These receptors can perceive their cognate microbial effector molecules leading to resistance. This layer of immunity is known as *R* gene-mediated resistance or effector-triggered immunity (ETI). It results in an incompatible interaction between host and an avirulent pathogen.

Activation of NB-LRR proteins launches a dramatic cellular reprogramming, involving accumulation of the stress hormone salicylic acid (SA) and reactive oxygen species within the cell and transcriptional reprogramming of pathogen-responsive genes. A hallmark of ETI, but not always observed, is a hypersensitive response (HR), which is typically associated with programmed cell death of infected cells. This local response is also able to prime uninfected tissue for subsequent attacks to a broad spectrum of pathogens, a phenomenon called systemic acquired resistance (SAR). It is essential for plants to tightly control their defense responses as it is deleterious for the host to activate defense inappropriately and thereby interrupting plants growth and reproduction cycle (Bostock, 2005).

R proteins can recognize effectors either directly by physical interaction or indirectly through their perturbation of host targets. A direct interaction of an effector with its cognate R protein is consistent with the gene-for-gene hypothesis proposed by Flor (1971). It is supposed to be a rare event given the fact that the Arabidopsis genome contains around 150 NB-LRR proteins compared to the high number of pathogen effectors plants encounter. An example for direct recognition is the physical interaction between the flax TIR-NB-LRR L6 and flax rust fungus effectors encoded by *AvrL567* genes (Dodds et al., 2006).

Indirect recognition has the advantage that R proteins can make use of the pathogen´s virulence strategy instead of recognizing specific patterns of individual pathogens and thereby bypass the evolutionary arms race against rapidly evolving pathogens. Three different modes for indirect recognition have been postulated (Dodds and Rathjen, 2010). In a first model, host proteins are guarded by NB-LRRs. By targeting host proteins, the effector activates R protein signaling through physical interaction (Dangl and Jones, 2001).

Alternatively, the effector modifies host proteins that are the virulence target or a structural mimic of the target. Modification of the target protein is recognized by R proteins (van der Hoorn and Kamoun, 2008). In a third model, the target protein is associated with the NB-LRR protein. Interaction of the effector with the target protein facilitates a subsequent recognition of the effector by the R protein (Collier and Moffett, 2009).

A well-characterized example of a host target guarded by NB-LRR proteins is the negative regulator of basal resistance RIN4 (RPM1 INTERACTING PROTEIN 4). RIN4 physically interacts with the R proteins RPM1 (RESISTANCE TO P. SYRINGAE PV. MACULICULA 1) and RPS2 (RESISTANCE TO P. SYRINGAE 2) and is targeted by the unrelated bacterial effectors AvrRpm1, AvrB and AvrRpt2 (Mackey et al., 2002; Kim et al., 2005). AvrRpm1 and AvrB induce RIPK (RPM1-INDUCED PROTEIN KINASE)-mediated phosphorylation of RIN4 which is recognized by the NB-LRR protein RPM1 resulting in its activation (Liu et al., 2011). In contrast, AvrRpt2 cleaves RIN4 which in turn leads to RPS2 activation (Mackey et al., 2003; Axtell and Staskawicz, 2003; Kim et al., 2005).

ETI can be described as an accelerated and amplified MTI response since both layers of immunity are using a highly overlapping signaling network (Tsuda et al., 2009). The major differences between MTI and ETI is derived by different timing and amplitude of the response (Katagiri and Tsuda, 2010).

1.2 EDS1 – master regulator in plant immunity

1.2.1 Identification and biochemical characterization

EDS1 (ENHANCED DISEASE SUSCEPTIBILITY1) constitutes a central regulatory hub in plant immunity. Initially, EDS1 was identified in a screen for new components involved in resistance mediated by the *RPP* (RESISTANCE TO P. PARASITICA) genes *RPP5* and *RPP14* towards the obligate biotrophic oomycete pathogen *Hyaloperonospora arabidopsidis* (Parker et al., 1996). *eds1* was shown to be a recessive mutation and homozygous *eds1* plants allowed heavy sporulation upon inoculation with *H. arabidopsidis* isolates that are recognized by known *RPP* genes in wild type plants (Parker et al., 1996). Furthermore, *eds1* exhibited enhanced susceptibility compared to wild type in compatible interactions. In contrast, other *R* gene-conditioned resistances were not compromised upon *Pseudomonas syringae* inoculation delivering the avirulence protein AvrB which is recognized by RPM1. It was concluded that EDS1 is necessary for resistance conferred by several but not all *R* gene responses (Parker et al., 1996). Shortly before, NDR1 (NON RACE-SPECIFIC DISEASE RESISTANCE1) was described to function as a common node downstream of pathogen perception by several R proteins, showing parallels to the observed requirements of EDS1 (Century et al., 1995). Later, a study by Aarts et al. (1998) revealed a correlation between the NB-LRR type of R proteins and their demand for either EDS1 or NDR1 in downstream signaling. R proteins with a N-terminal TIR domain depend predominantly on EDS1 whereas R proteins belonging to the class with a N-terminal CC motif typically require

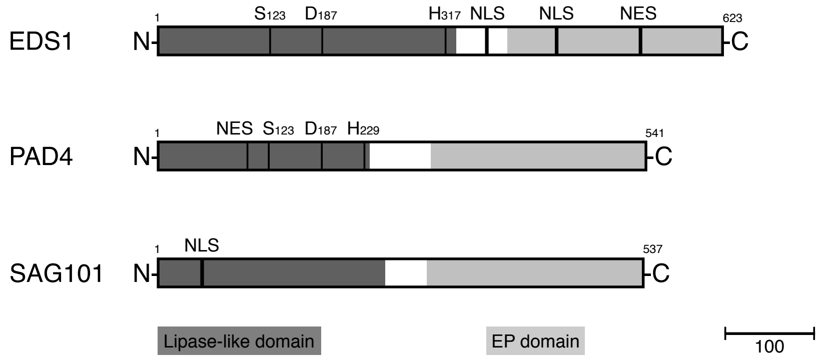

Figure 1.1: Schematic representation of the domain structure of Arabidopsis EDS1, PAD4 and SAG101 proteins. The lipase-like domain is shown in dark grey and the EP domain in light grey. A putative catalytic triad in EDS1 and PAD4 sequences composed of serine (S), aspartate (D) and histidine (H) residues is highlighted in the lipase-like domain. EDS1 predicted bipartite NLS at amino acid positions 366 and 440 and a putative NES at position 528 are marked. SAG101 protein sequence contains a predicted monopartite NLS in amino acids 48 – 51. Numbers indicate amino acid positions.

NDR1 to trigger resistance.

EDS1 and its sequence-related interaction partners PHYTOALEXIN DEFICIENT4 (PAD4) and SENESCENCE ASSOCIATED GENE101 (SAG101) share a so-called "EP" (EDS1/PAD4) domain of unknown function at their C-termini, defining this protein family (Feys et al., 2001, 2005). Sequence analysis of EDS1 showed similarities to eukaryotic lipases on the N-terminal half, including a serine-aspartate-histidine catalytic triad implying hydrolase activity (Falk et al., 1999; Wagner et al., 2011) (Figure 1.1). Even though the catalytic triad is conserved in all EDS1 and PAD4 orthologs among vascular plants, no lipase activity has been reported so far. Monocotyledons do not possess TIR-NB-LRRs but orthologs of PAD4 and EDS1, likely reflecting an ancestral function of EDS1 and PAD4 in basal immunity. In contrast, SAG101 is only expressed in dicotyledonous plants, suggesting a co-appearance with TIR-NB-LRR-mediated resistance.

EDS1 is a soluble nucleo-cytoplasmic protein that forms molecularly and spatially distinct complexes with PAD4 and SAG101 (Feys et al., 2005; Rietz et al., 2011). The EDS1/PAD4 complex exhibits nucleo-cytoplasmic distribution, whereas EDS1/SAG101 is found in the nucleus due to SAG101 predominant presence in this compartment (Feys et al., 2005). According to binding affinity measurements in yeast three-hybrid assays, EDS1 prefers to build homodimers, mostly present in the cytoplasm, over binding to PAD4 or SAG101 (Rietz et al., 2011).

Studies by Zhu et al. (2011) suggest a ternary complex consisting of EDS1, PAD4 and SAG101. They found a requirement for EDS1 for PAD4/SAG101 interaction. This result was obtained by bimolecular fluorescence complementation (BiFC) assays and confirmed by co-immunoprecipitation upon transient *Agrobacterium*-mediated expression of EDS1, PAD4 and SAG101 in *Nicotiana benthamiana*. So far, no ternary complex could be detected *in vivo* in Arabidopsis (Rietz et al., 2011). Analysis of a recently obtained crystal structure of the EDS1/SAG101 heterodimer suggests that a ternary complex of the three proteins is rather unlikely (S. Wagner, personal communication). Hence, there are still open questions concerning the composition of EDS1 complexes with PAD4 and SAG101. Further structure analysis might help to reveal putative specific functions of the various EDS1/PAD4/SAG101 complexes in plant immunity.

1.2.2 Molecular functions of EDS1 complexes in immunity

Early studies revealed EDS1 contribution in basal immunity and its essential role in TIR-NB-LRR-mediated resistance (Parker et al., 1996; Aarts et al., 1998). A well-characterized example for EDS1-dependent TIR-NB-LRR-conditioned resistance is the signaling pathway triggered by RPS4 (RESISTANT TO PSEUDOMONAS SYRINGAE 4) activation (Wirthmueller et al., 2007). The TIR-NB-LRR protein RPS4 is able to recognize the bacterial effector AvrRps4 (Hinsch and Staskawicz, 1996) which is secreted into the plant cell by *P. syringae* DC3000 via the bacterial type III secretion system. Inside the cell, AvrRps4 is cleaved by a plant protease (Hinsch and Staskawicz, 1996; Sohn et al., 2009). This cleavage is required for its virulence but not for its avirulence activity (Sohn et al., 2009). RPS4 signaling upon effector recognition was shown to be entirely EDS1-dependent (Wirthmueller et al., 2007). It was demonstrated that EDS1 acts downstream of TIR-NB-LRR activation but upstream of defense gene expression, production of SA and host cell death (Feys et al., 2001; Zhang et al., 2003; Wirthmueller et al., 2007).

Furthermore, EDS1 and PAD4 were shown to be required for runaway cell death observed in *lsd1* (LESION SIMULATING DISEASE 1) mutants (Rustérucci et al., 2001). *LSD1* is a negative regulator of cell death and *lsd1* mutants fail to limit spreading of programmed cell death in response to superoxide treatment or upon infection with pathogens (Dietrich et al., 1997; Rustérucci et al., 2001). EDS1 and PAD4 are able to transduce reactive oxygen species signaling induced by photo-oxidative stress or during immune response, leading to cell death (Rustérucci et al., 2001; Mateo and Mühlenbock, 2004; Mühlenbock et al., 2008). This implicates a contribution of EDS1 and PAD4 in redox signaling upon abiotic and biotic stress. Moreover, work by Straus et al. (2010) indicates a ‘master‘ role of EDS1 in coordinating interaction of reactive oxygen species and SA to control initiation and spreading of cell death. Both examples suggest an involvement of redox components in EDS1 signaling.

1.2.3 EDS1 signals together with PAD4 and SAG101

EDS1 cooperates closely with its interaction partners PAD4 and SAG101 (Feys et al., 2001, 2005). Genetically, *PAD4* and *SAG101* are partially redundant. Loss of *SAG101* can be compensated for by *PAD4* in basal and *R* gene-triggered immunity. In contrast, absence of *PAD4* is not fully compensated for by *SAG101*, indicating a unique capability of *PAD4* possibly due to its nucleo-cytoplasmic localization. *pad4*/*sag101* double mutants are completely disabled in basal and TIR-NB-LRR-mediated resistance, similar to *eds1* (Feys et al., 2005). This suggests an intrinsic signaling function of both proteins in immunity additional to their demonstrated role in stabilizing EDS1 (Feys et al., 2005; Wiermer et al., 2005).

Apart from its contribution to basal and *R* gene-mediated resistance, PAD4 is essential for defense against green peach aphids (*Myzus persicae*) (Pegadaraju et al., 2005). Activity of PAD4 in immunity against aphids was shown to be independent of *EDS1* and pathogen-induced SA (Louis et al., 2012). Mutation of the conserved serine in the predicted lipase catalytic triad of *PAD4* partially compromised defense against aphids. In contrast, defense against microbial pathogens was not affected by the mutation. This indicates a requirement of different PAD4 molecular functions in defense responses against pathogens and aphids (Louis et al., 2012).

EDS1 fulfills different roles in pathogen resistance in complex or dissociated from PAD4. Work of Rietz et al. (2011) showed a requirement of EDS1 bound to PAD4 in basal resistance. Pathogen proliferation of virulent *H. arabidopsidis* isolates and *P. syringae* strains was enhanced in stable transgenic plants carrying a single amino acid exchange in *EDS1* (eds1L262P). These mutants failed to bind PAD4 but retained interaction with SAG101. Moreover, induction of PR1 and SA accumulation was disabled upon *P. syringae* DC3000 infection in eds1L262P plants and they were compromised in SAR. In contrast, EDS1 dissociated from PAD4 was able to activate TIR-NB-LRR-triggered cell death in response to *H. arabidopsidis*. Cell death induction was shown to be independent of SAG101, suggesting that SAG101 does not compensate PAD4 activity in this defense output (Rietz et al., 2011).

1.2.4 EDS1 nuclear-cytoplasmic shuttling and balance contribute to immunity

EDS1 amino acid sequence contains two predicted bipartite nuclear localization signals (NLS) and one putative nuclear export signal (NES) (Falk et al., 1999; García et al., 2010) (Figure 1.1). However, mutations of core residues in these sequences do not alter protein distribution (García et al., 2010). García et al. (2010) demonstrated an active transport of EDS1 between cytoplasm and nucleus through nuclear pores by nuclear transport receptor-mediated shuttling.

Several studies over the last years highlighted the importance of nucleo-cytoplasmic shuttling of immune regulators via the nuclear pore complex (NPC) to control defense responses in plants. A screen to identify components of constitutive resistance caused by a gain-of-function mutation in the TIR-NB-LRR protein SNC1 (SUPPRESSOR OF NPR1-1, CONSTITUTIVE) revealed mutations in genes encoding proteins belonging to the nucleo-cytoplamic trafficking machinery, thereby providing first evidence for involvement of nuclear pore complex-mediated shuttling in immunity (Li et al., 2001; Zhang et al., 2005). *Snc1* carries a missense mutation leading to enhanced disease resistance accompanied by constitutive expression of pathogen-related (PR) genes, increased levels of the phytohormone SA and a dwarf morphology. *Snc1* phenotypes can be suppressed to different degrees by *mos* (MODIFIER OF SNC1) mutants. The NPC is composed of nucleoporin proteins (Nups) and nuclear import is initiated by the interaction between cargo proteins containing a NLS, and the transport receptors Importin α and Importin β in the cytoplasm. *MOS6* was found to encode importin α3, the Arabidopsis homolog to human nucleoporin 96 (Palma et al., 2005). Additionally, *MOS3* and *MOS7* are homologs of yeast and vertebrate genes encoding the nucleoporines *Nup96* and *Nup88*, respectively (Zhang et al., 2005; Cheng et al., 2009).

Interestingly, components of the animal NPC were also found to contribute to immunity, as e.g. demonstrated for the mouse *Nup96* and *Drosophila Nup88*, providing evidence that involvement of certain *Nups* in immunity is conserved (Uv et al., 2000; Faria et al., 2006).

mos6, *mos3* and *mos7* single mutants exhibited increased susceptibility and partially suppressed dwarfism in the *snc1* background, emphasizing the essential function of nucleocytoplasmic trafficking in plant innate immunity (Palma et al., 2005; Zhang et al., 2005; Cheng et al., 2009). Furthermore, plants with defects in *MOS7* accumulated reduced levels of EDS1 and the transcriptional co-activator NPR1 (NONEXPRESSOR OF PR GENES) in unchallenged leaves (Cheng et al., 2009). These changes are not reflected on the transcript level, suggesting an effect of *mos7* in protein synthesis or stability. In contrast, snc1 protein level is not altered in *snc1*/*mos7* double mutants. However, there were changes in subcellular distribution shifting the balance from elevated nuclear snc1 in *snc1* single mutants to increased cytosolic snc1 accumulation in *snc1*/*mos7* plants (Cheng et al., 2009). These results suggest that the concentration of specific immune regulators might be fundamental for defense regulation.

More recently, further members of the conserved Nup107-160 nuclear pore sub-complex were identified to contribute to basal and TIR-NB-LRR-mediated resistance. *nup160* and *seh1* are selectively impaired in basal immunity and resistance conditioned by TIR-NB-LRRs but unaffected in resistance triggered by CC-NB-LRR proteins (Wiermer et al., 2012). This indicates a specific contribution of these components of the nuclear pore complex to certain defense signaling pathways. In contrast, *mos7* plants showed defects in basal and NB-LRR-mediated resistance with varying degrees of susceptibility depending on the R protein, but did not discriminate between TIR- or CC-NB-LRR-triggered resistance (Cheng et al., 2009). Furthermore, there is an increase of nuclear poly(A) mRNA in *nup160* and *seh1* (Wiermer et al., 2012). Together with the observation of a selective decrease in *EDS1* transcript and protein levels in *nup160*, a need for *Nup160* for nuclear mRNA export and complete expression of EDS1-mediated resistance was hypothesized (Wiermer et al., 2012). Taken together, these results suggest that the nuclear pore trafficking machinery is an essential component in regulating immunity and contributes to EDS1 accumulation.

Following this line, analysis of EDS1 accumulation upon inoculation with pathogens triggering TIR-NB-LRR-dependent resistance revealed a post-transcriptionally-controlled increase of nuclear EDS1 early after pathogen challenge (García et al., 2010). This conclusion is based on the observation that there is an early rise of nuclear EDS1 amounts before changes occur at the transcript level. Later during defense signaling, there is also an increase in *EDS1* induced by a positive feedback loop (Wiermer et al., 2005; García et al., 2010). Changes of nuclear EDS1 became equilibrated with the cytoplasmic pool at later time points (García et al., 2010). Plants with enforced cytosolic EDS1, either by an attachment of a nuclear localization signal (NES) or by fusion to a glucocorticoid receptor hormone binding domain (EDS1-GR) that allowed conditional release of EDS1 into the nucleus upon Dexamethasone (Dex) treatment, were compromised in basal and *R* gene-mediated resistance (García et al., 2010). Additionally, expression of EDS1-dependent defense genes was impaired in lines with enforced cytosolic EDS1 accumulation upon challenge with the virulent *P. syringae* strain DC3000 expressing the effector AvrRps4. EDS1-GR lines displayed expanded HR in the absence of Dex after infection with an avirulent *H. arabidopsidis* isolate (García et al., 2010). Based on these results, García et al. (2010) hypothesized that cytoplasmic and nuclear pools of EDS1 are required for a complete resistance response including restriction of cell death at the infection site. Moreover, an essential role of nuclear EDS1 in defense-related transcriptional reprogramming was shown. It was speculated that cytosolic and nuclear EDS1 pools communicate through nuclear pores to coordinate resistance (García et al., 2010).

1.2.5 EDS1 forms complexes with TIR-NB-LRR proteins

Recently, studies of Heidrich et al. (2011) and Bhattacharjee et al. (2011) demonstrated complex formation of EDS1 with the TIR-NB-LRR proteins RPS4, RPS6 and SNC1 additional to an association with the negative regulator of plant immunity SRFR1 (SUPPRESSOR OF rps4-RLD1). SRFR1 is a tetratricopeptide repeat protein that dampens immunity by altering NB-LRR stability and the expression of their coding genes (Li et al., 2010; Kim et al., 2010). Moreover, EDS1 was shown to interact with two sequence-unrelated bacterial effector proteins, AvrRps4 and HopA1 (Bhattacharjee et al., 2011). Presence of the effectors disrupted EDS1 interaction with the TIR-NB-LRR proteins and SRFR1 (Bhattacharjee et al., 2011).

Based on these findings, the authors suggested that EDS1 might be a common virulence target guarded by several TIR-NB-LRR proteins (Heidrich et al., 2011; Bhattacharjee et al., 2011). They proposed a model in which EDS1, together with PAD4 and SAG101 as a vulnerable regulatory hub in basal immunity, is targeted by pathogen effectors to disable its function in activating basal resistance. Their data indicates that perturbation of EDS1 complexes with TIR-NB-LRR proteins by effectors leads to an activation of TIR-NB-LRR signaling. This data supports the idea that a key function of EDS1 in *R* gene-mediated resistance derives from being guarded by TIR-NB-LRR proteins. Thereby, EDS1 would directly connect MTI with ETI signaling. Since it was previously shown that EDS1 acts downstream of TIR-NB-LRR proteins (Zhang et al., 2003; Wirthmueller et al., 2007), a possible scenario was proposed in which EDS1 has an ancient signaling function in basal immunity which later in evolution became co-opted in *R* gene-mediated resistance (Heidrich et al., 2011; Bhattacharjee et al., 2011).

Intriguingly, the proposed scenario is consistent with the results of Rietz et al. (2011), pointing to a role of EDS1 dissociated of PAD4 in R protein-mediated cell death. In contrast, EDS1 bound to PAD4 is necessary for basal resistance. Thus, EDS1 alone might be an effector target guarded by TIR-NB-LRR proteins (Bhattacharjee et al., 2011).

Interestingly, a recent study revealed an interaction of PAD4 with a newly identified TIR-NB-LRR protein, providing evidence for an association between PAD4 and TIR-NB-LRR proteins for the first time (Kim et al., 2012). In previous studies, an association of PAD4 with RPS4 and RPS6 was tested and could not be detected (Bhattacharjee et al., 2011). EDS1 was also shown to interact with this TIR-NB-LRR protein in *Nicotiana benthamiana* transient expression assays. The *TIR-NB-LRR* gene *VICTR* (VARIATION IN COMPOUND TRIGGERED ROOT growth response) was discovered by a chemical genetic screen (Kim et al., 2012). It is responsible for root growth arrest in Col-0 in response to the small chemically synthesized molecule DFPM (Kim et al., 2011, 2012). In a previous study, it was shown that DFPM activates *R* gene-mediated immune signaling, leading to disruption of the ABA signaling pathway (Kim et al., 2011). DFPM-induced signaling required EDS1, PAD4 and the co-chaperones RAR1 (REQUIRED FOR Mla12 RESISTANCE) and SGT1b (SUPPRESSOR OF G-TWO ALLELE OF SKP1b), which are involved in R protein stabilization (Kim et al., 2011).

By BiFC and co-immunoprecipitation assays, an interaction of VICTR with EDS1 and PAD4 was detected in the nucleus. Further analysis including an EDS1 variant incapable of binding PAD4 revealed that EDS1 dissociated from PAD4 is also able to form a complex with VICTR (Rietz et al., 2011; Kim et al., 2012). This suggests that different EDS1 domains are important for associating with either PAD4 or TIR-NB-LRR proteins.

DFPM-induced VICTR-mediated root growth arrest, which depends on EDS1 and PAD4, indicates a link between R protein signaling and root growth arrest (Kim et al., 2012). Based on these findings, a model was proposed in which root growth is stopped upon recognition of soil-born pathogens (Kim et al., 2012). This work points for the first time to EDS1 playing a role in immunity towards root pathogens in contrast to most studies analyzing EDS1 signaling in the context of leaf pathogens.

1.2.6 SA signaling within the EDS1 defense pathway

EDS1, together with PAD4, stimulates production of the defense hormone SA (Zhou et al., 1998; Feys et al., 2001). SA is known to play a central role in resistance against biotrophic- and hemibiotrophic pathogens which complete at least parts of their life cycle on living plant tissue (Vlot et al., 2009). The importance of SA in activating defense is emphasized by the observed compromised resistance in mutants impaired in SA production (Gaffney et al., 1993; Hunt et al., 1997; Wildermuth et al., 2001a). Pathogen-induced SA is synthesized via the chloroplast-localized protein ICS1 (ISOCHORISMATE SNYTHASE 1) (Wildermuth et al., 2001a). EDS1 and PAD4 regulate accumulation of SA as part of a positive feedback loop. Initial activation of EDS1, together with PAD4, induces defense gene expression including upregulation of *ICS1* that is crucial for defense-related SA accumulation (Wildermuth et al., 2001a; Attaran et al., 2009; García et al., 2010). Besides, SA contributes to *EDS1* and *PAD4* expression together with other defense-related genes, resulting in defense amplification (Jirage et al., 1999; Feys et al., 2001).

By regulating SA activity, EDS1 is indispensable for SAR (Malamy et al., 1990; Métraux et al., 1990). A key component of SAR is the transcriptional co-activator NPR1. NPR1 contains an ankyrin-repeat motif and a BTB/POZ domain (Cao et al., 1997). It is able to interact with several members of the TGA family of bZIP transcription factors to activate PR gene expression (Zhang et al., 1999; Zhou et al., 2000). Additionally, NPR1 induce expression of other transcription factors with activating or suppressing functions (Wang et al., 2006). Over the last years, NPR1 activity was studied in depth and revealed a complex regulatory mechanism of NPR1 to fine-control immunity. In uninduced cells, NPR1 oligomers, hold together by disulfide bounds, are mostly retained in the cytosol (Mou et al., 2003). NPR1 monomers reaching the nucleus become degraded by the proteasome, thereby preventing its role as a transcriptional co-activator (Spoel et al., 2009). Upon pathogen challenge, SA levels increase, resulting in redox changes within the cell. NPR1 oligomers disassemble due to reduction of disulfide bounds, leading to an increase of monomeric NPR1. NPR1, containing a bipartite nuclear localization sequence, can enter the nucleus and promotes defense gene induction (Mou et al., 2003). Interestingly, X. Dong and colleagues found that inhibiting degradation of NPR1 after SAR induction compromises NPR1-dependent gene expression (Spoel et al., 2009). Based on this result, it is postulated that proteasome-mediated turnover of NPR1 is needed for full target gene expression (Spoel et al., 2009). A model is proposed stating that "exhausted" NPR1 has to be cleared from its site of action to allow "fresh" NPR1 to re-initiate transcription (Spoel et al., 2009). Furthermore, two paralogs of NPR1, NPR3 and NPR4, were identified as receptors of SA. NPR3 and NPR4 are involved in NPR1 degradation by the proteasome in a SA-regulated manner (Fu et al., 2012).

Similar regulation of immune-related transcriptional activators was shown in the animal field. In animals, transcriptional induction of defense genes is regulated by the transcription factor NF-κB (NUCLEAR FACTOR-κB). In uninduced cells, NF-κB is sequestered in the cytoplasm by its cofactor I-κB. In response to pathogens, I-κB becomes degraded by the proteasome and NF-κB is released into the nucleus to activate defense gene expression (Verma et al., 1995).

1.3 Thesis aims

Even though we gained deeper insights into different aspects of Arabidopsis EDS1 signaling over the last years, the precise molecular function of EDS1 during plant defense activation still remains elusive. It is known that EDS1 operates downstream of or coincides with TIR-NB-LRR activation but upstream of cell death initiation, accumulation of reactive oxygen species and the stress hormone SA as well as defense gene expression (Wirthmueller et al., 2007). Thereby, it connects pathogen perception with activated defense responses. The aim of this study was to understand how EDS1 and its interacting partners PAD4 and SAG101 coordinate the multiple defense outputs. Previous studies showed that EDS1 is able to shuttle between subcellular compartments (García et al., 2010). It forms molecularly and spatially distinct complexes with PAD4 and SAG101, indicating different functions of the various complexes in the cytosol and nucleus (Feys et al., 2005). Cytoplasmic and nuclear functions of EDS1 were hypothesized to cooperate in mediating a complete and balanced immune response. It was demonstrated that nuclear EDS1 is required for TIR-NB-LRR-conditioned transcriptional reprogramming (García et al., 2010).

To further discriminate between nuclear and cytoplasmic function of EDS1, I made use of stable transgenic Arabidopsis lines expressing EDS1 which is forced into the nucleus. Cellular and molecular biological approaches as well as biochemical characterization of plants with EDS1 restricted to the nucleus by fusion to a strong (SV40 viral) nuclear localization signal (EDS1-NLS) will allow me to reveal functional significance of nuclear EDS1.

In addition, to determine immediate effects of nuclear EDS1 and connecting these to gene expression, transgenic plants expressing EDS1-NLS under the control of a conditional estradiol-inducible promoter were analyzed.

To examine the role of the EDS1 signaling partners PAD4 and SAG101 for EDS1 function in the nucleus, knockout lines of *PAD4* and *SAG101* or both in the EDS1-NLS background were generated and analyzed for their resistance phenotypes. To assess the impact of SA biosynthesis and signaling on nuclear EDS1 activity, mutants in these pathways were explored in genetic studies.

Furthermore, EDS1-NLS lines were used to find new nuclear EDS1 cooperation partners by mass spectrometry analysis with the aim to discover putative associations of EDS1 with components involved in transcriptional reprogramming.

2 Results

2.1 Developmental effects of EDS1 mislocalization to the nucleus

EDS1 localizes to the cytoplasm and nucleus in plant cells (Feys et al., 2005). Previous studies showed when EDS1 is excluded from the nucleus, plants are partially compromised in basal defense and *R* gene-mediated resistance. These results suggest a contribution of cytoplasmic and nuclear EDS1 activities for resistance (García et al., 2010). Based on these findings and the observation that the nuclear EDS1 pool rises early upon infection but becomes equilibrated after a short period of time, it was hypothesized that a fine balance of both pools is important for EDS1 function in resistance. García et al. (2010) also demonstrated a requirement of nuclear EDS1 and/or its active shuttling through the nuclear pore complex for transcriptional reprogramming upon pathogen challenge.

To analyze the role of nuclear EDS1 for defense activation and resistance, transgenic lines were generated where EDS1 is forced into the nucleus by fusion to a strong nuclear localization sequence (NLS) (García, 2009). The NLS sequence is derived from the simian virus 40 (SV40) large T antigen (PKK128KRKVGG) (Kalderon et al., 1984) and attached to the C-terminus of EDS1. A yellow fluorescent protein (YFP) sequence was fused between EDS1 and the NLS to visualize localization of the tagged protein by confocal laser-scanning microscopy (CLSM). The coding sequences are driven by the native *EDS1* promoter (pEDS1:gEDS1-mYFP-NLS) and stably expressed in the Col-0 *eds1-2* mutant background, hereafter referred to as EDS1-YFP-NLS.

Additionally, control transgenic lines were generated expressing EDS1-YFP attached to a mutated, non-functional nuclear localization sequence (nls, PKT128KRKVGG). Selected EDS1-YFP-nls lines expressing similar EDS1 levels as Col-0 were found in the cytoplasm and nucleus. However, EDS1-YFP-nls did not complement hypersusceptibility of *eds1-2* and showed significant transcriptional reprogramming in unchallenged plants compared to Col-0 (Supplementary figure 5.1), rejecting it as an suitable wild type (wt)-like control line. Therefore, EDS1-YFP-nls was not further used as a control in this study. Instead, a stable transgenic line was chosen expressing EDS1 fused to YFP at its C-terminus in the Col-0 *eds1-2* background, referred to as EDS1-YFP. The transgene is driven by the native *EDS1* promoter. EDS1-YFP has higher cytoplasmic and nuclear EDS1 protein levels than wt plants (Figure 2.4). This was important since the main EDS1-YFP-NLS line characterized in this study expresses similar EDS1 protein amounts as EDS1-YFP. Potential impacts of high nuclear amounts of YFP could be ruled out by using this control.

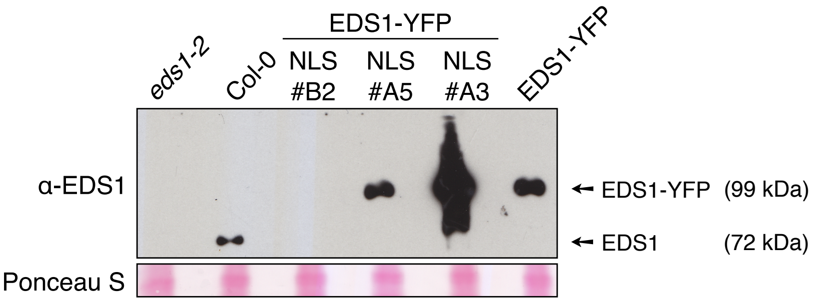

Figure 2.1: EDS1-YFP protein accumulation. Immunoblot analysis was performed on total leaf extracts of EDS1-YFP-NLS independent transgenic lines. Protein extracts were prepared from 3-week-old plants. The membrane was probed with α-EDS1 antibody. Ponceau S staining was performed to ensure equal sample loading. Migration of wt EDS1 and EDS1-YFP-NLS is marked by arrows on the right.

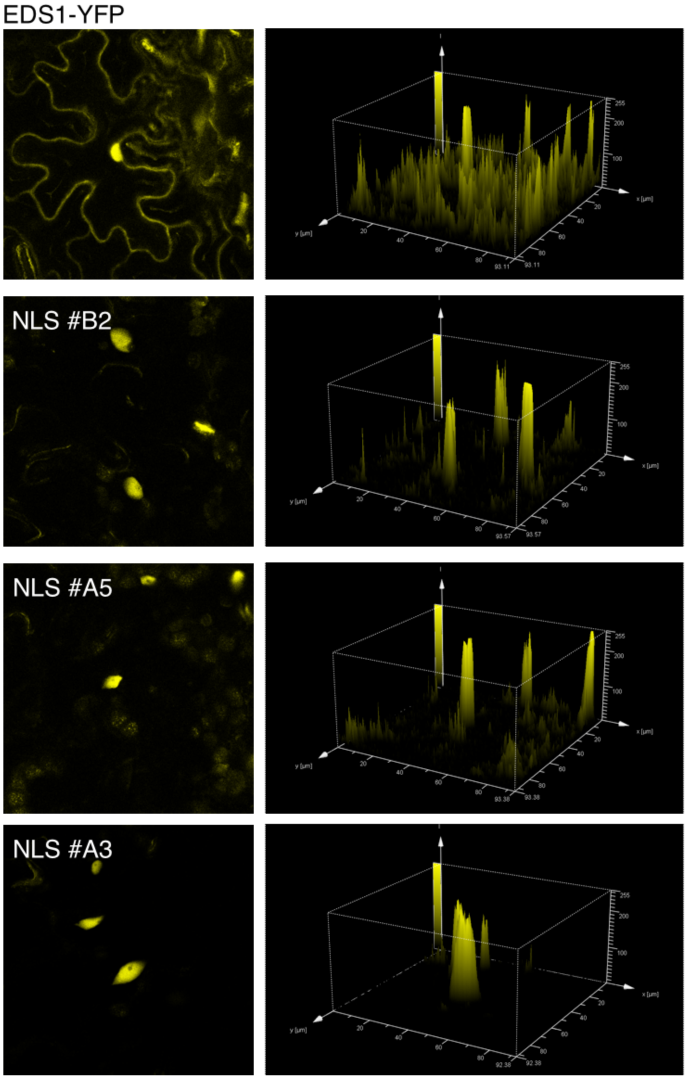

Figure 2.2: Enforced localization of EDS1 to the nucleus. Confocal images of YFP fluorescence in leaf epidermal tissue of EDS1-YFP and EDS1-YFP-NLS lines. Images were taken of 3-week-old plants. Right panel shows 3D plot of the image in the left panel.

Multiple independent lines with various levels of EDS1-YFP-NLS protein were selected in a range from less total protein accumulation to highly increased levels compared to EDS1 expression in Col-0 (Figure 2.1). The expected nuclear localization of EDS1-YFP-NLS protein in all three selected lines was confirmed by CLSM (Figure 2.2). Interestingly, two out of three selected lines started to show growth inhibition when grown under normal conditions (22°C, short day, 60 % humidity) at different stages of plant development (Figure 2.3). Line EDS1-YFP-NLS #A3 had the most striking developmental phenotype; it exhibited stunted growth from germination on, leaves became chlorotic and plants were dying after around 2 weeks. Line EDS1-YFP-NLS #A5 displayed regular growth until plants were 4- to 5-week-old. At that time, leaves started to curl and plants looked compressed. In week 6 to 7, EDS1-YFP-NLS #A5 began to show chlorosis, developing into necrosis. #A5 plants stayed dwarf but were able to produce some siliques with few seeds. Line EDS1-YFP-NLS #B2 development was not affected and grew wt-like throughout its whole lifecycle. It expressed the lowest EDS1 protein level of all selected lines. Control line EDS1-YFP and *eds1-2* knock-out plants displayed wt-like development at 22°C.

By comparing differences in the strength of developmental phenotypes with the amount of expressed nuclear EDS1 protein in all three lines, there was a noticeable correlation between protein level and severity of the growth inhibition. Line EDS1-YFP-NLS #A3 expressed by far the highest amount of EDS1 protein and showed growth retardation starting from early development, whereas #A5 with lower EDS1 level as #A3 but higher than Col-0 showed less severe growth inhibition. Line #B2, with EDS1 levels less than in Col-0, displayed wt-like development. This observation suggests that a certain threshold of nuclear EDS1 in the absence of cytoplasmic EDS1 has to be passed before developmental abnormalities occur.

To analyze if growth inhibition is caused by simply too much nuclear EDS1, I compared EDS1 levels in the nucleus of line #B2 and #A5 with Col-0 and EDS1-YFP. By assessing the amount of nuclear EDS1 in #A5 and EDS1-YFP, I observed at least the same EDS1 level in EDS1-YFP as in #A5 (Figure 2.4). Line EDS1-YFP did not show any growth abnormalities, leading to the conclusion that high amounts of nuclear EDS1 cannot be the reason for developmental abnormalities. This implies that high and unbalanced accumulation of EDS1 in the nucleus results in growth inhibition of plants. Nuclear EDS1 amounts of #B2 were still slightly reduced related to Col-0, but to a much lower extent compared to total levels in both plants, supporting the expectation that all EDS1 protein in #B2 localized to the nucleus.

It has to be noted that after nuclear fractionation, there were also elevated levels of EDS1 protein in the nuclear-depleted fraction, which is in contrast to the confocal microscopy analysis (Figure 2.2). Since confocal analysis of numerous Z stacks of lines EDS1-YFP-NLS #A3, #A5, and #B2 showed only nuclear localization of EDS1 protein, I assume leakage of EDS1 from the nucleus into the cytoplasmic pool during biochemical fractionation. Another reason for EDS1 detection in the nuclear-depleted fraction might be due to protein synthesis. Proteins are synthesized at the ribosomes in the cytoplasm before they are transported to their destination. This means that at least some EDS1-YFP-NLS protein is present in the cytoplasm before it is taken up by the nuclear trafficking machinery and shuttled into the

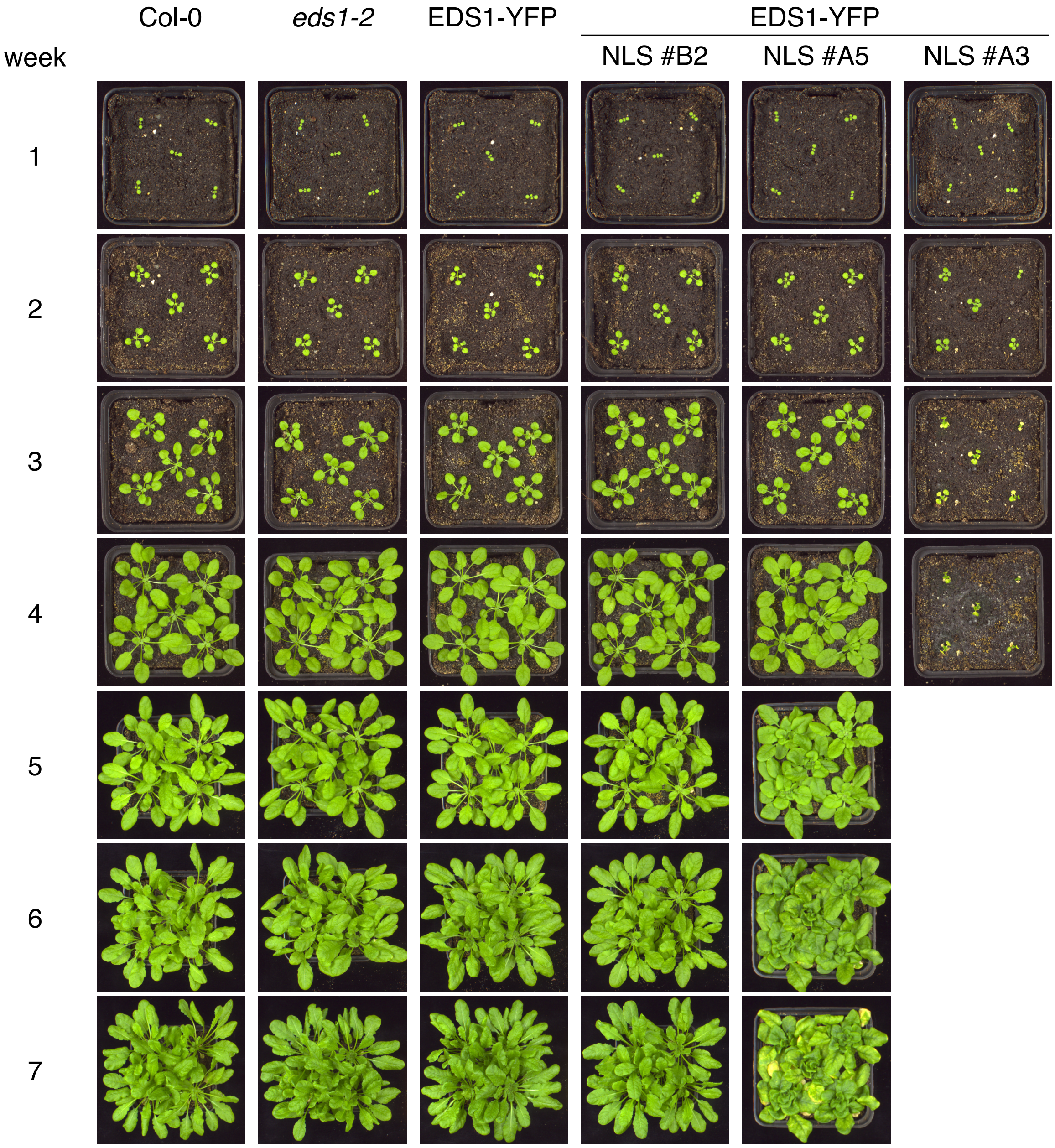

Figure 2.3: Growth phenotype of EDS1-YFP-NLS independent transgenic lines. Plant development of EDS1-YFP-NLS lines at 22°C. Pictures were taken at the indicated time points after sowing.

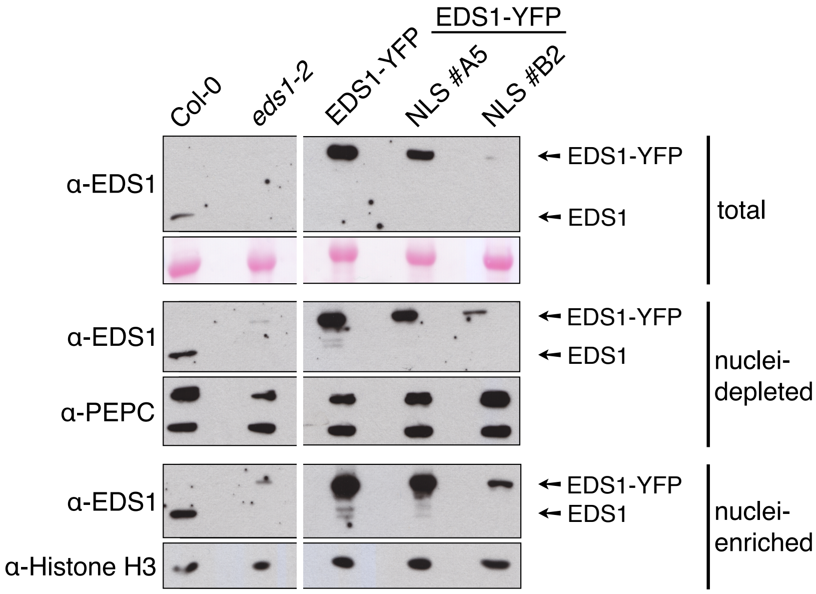

Figure 2.4: Accumulation and subcellular distribution of EDS1-YFP fusion protein in stable transgenic lines. Protein extracts were prepared from leaf tissues of 3-week-old healthy plants. Protein gel blot analysis of EDS1 in total (top panel), nuclei-depleted (middle panel) and nuclei-enriched (bottom panel) fractions. PEPC-C and Histone H3 were used as cytosolic and nuclear marker, respectively. Ponceau S staining of the membrane indicates equal loading and transfer onto the membrane. wt EDS1 protein and EDS1-YFP-NLS bands are labeled on the right. Localization analysis was performed three times in independent experiments with similar results.

nucleus. The relatively high amounts of EDS1-YFP-NLS protein detected in the nuclear-depleted fraction compared to the nuclear-enriched fraction (16x enriched) is in clear conflict with the CLSM analysis. Therefore, I favor the explanation that the fractionation procedure is the major reason for detection of EDS1-YFP-NLS protein in the cytoplasm. Probably some of the nuclei were destroyed during grinding and parts of the soluble nuclear protein was released into the nuclear-depleted fraction, whereas histones, bound to large chromatin structures, did not leak (Figure 2.4). The suitability of a fractionation method always depends on the protein of interest. Therefore, it might be worth testing further fractionation protocols to confirm nuclear localization of EDS1-YFP-NLS by molecular biology approaches.

In summary, high accumulation of nuclear EDS1 in the absence of cytosolic EDS1 resulted in growth inhibition of plants. Furthermore, a threshold of nuclear EDS1 accumulation appears to be passed before developmental abnormalities occurred. Alternatively, suppression of nucleo-cytoplasmic shuttling, prohibited by the NLS fusion, may induce growth defects in these plants.

2.2 Investigation of EDS1-YFP-NLS defense outputs

Growth inhibition is a common characteristic of plants exhibiting constitutive resistance (Bowling et al., 1994, 1997; Li et al., 2001; Shirano et al., 2002). Thus, the described growth defects of line EDS1-YFP-NLS #A5 resemble Arabidopsis mutants with induced defense responses. EDS1 is a major player in plant immunity and constitutive resistance phenotypes of autoimmune-mutants were abolished in *eds1-2* knock-outs (Li et al., 2001; Clarke et al., 2001; Shirano et al., 2002; Zhou et al., 2004). This prompted me to test if line #A5 shows activated defense without pathogen trigger.

2.2.1 Transcriptional regulation

Recognition of pathogens by host plants induces a broad transcriptional reprogramming of defense-related genes. To study if EDS1-YFP-NLS #A5 plants display transcriptional regulation similar to pathogen-infected plants, a gene-expression microarray analysis was performed on 4-week-old #A5 plants and Col-0 (done by Ana Garcìa). At this developmental stage, #A5 plants did not show morphological abnormalities yet. Expression analysis was carried out by using an Affymetrix ATH1 Genome Array Chip including 3 technical replicates of each line.

Evaluation of differences revealed massive transcriptional reprogramming in #A5 compared to Col-0 (> 2000 genes, Figure 2.5A). 57 % (1188) of the differentially regulated genes were induced and 43 % (904) repressed. These broad changes in gene expression point to an alteration affecting various plant biological processes. For a closer examination of the gene classes changed in #A5, a Gene Ontology (GO) term analysis was performed. By this, differentially regulated genes were categorized according to their biological function. Many of the differentially regulated genes in #A5 are responsive to stimuli in general (e.g. stress, endogenous or hormone stimuli) or related to biotic and abiotic stresses (Figure 2.6A). A deeper examination of genes which were > 10-fold induced (106 genes) showed that most are associated with plant defense or defense signaling processes (Figure 2.6B). This suggests a predominant induction of defense-related genes in #A5. By contrast, most of the repressed genes were assigned to processes related to carbohydrate metabolism and photosynthesis (Figure 2.6C). It is broadly accepted that defense activation in plants comes at a cost of plant performance (Tian et al., 2003; Alcázar and Parker, 2011). Thus, down regulation of primary metabolic processes as well as photosynthesis activity for the benefit of defense responses agrees with such reprogramming. In summary, GO term analysis suggests that genes changed in #A5 compared to Col-0 are mainly related to biotic stress responses, emphasized by the finding that these genes were barely assigned to any other major biological process. Even though the observed growth defects in #A5 might point to genes involved in plant development, no particular changes in previously known developmental regulator genes were identified (M. Branchat, personal communication).

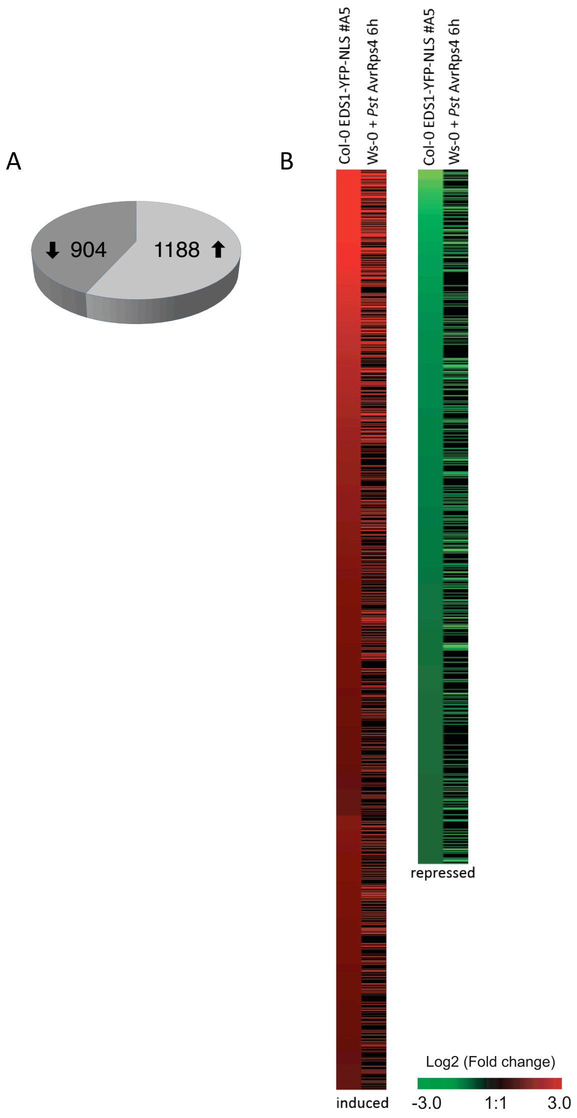

Figure 2.5: Analysis of differentially regulated genes in EDS1-YFP-NLS #A5. (A) Number of repressed and induced genes (at least 2-fold changes, p-value < 0.01) in EDS1-YFP-NLS #A5 compared to Col-0. **(B)** Clustering of all differentially regulated genes (at least 2-fold changes) in EDS1-YFP-NLS #A5 *vs.* wt Col-0 and in Ws-0 plants infiltrated with *Pst*AvrRps4 at 6h (*vs.* mock-inoculation), displayed as heatmaps. Heatmaps were generated using the Genesis software (http://genome.tugraz.at). Colors refer to $\log_2$ fold-change, as indicated.

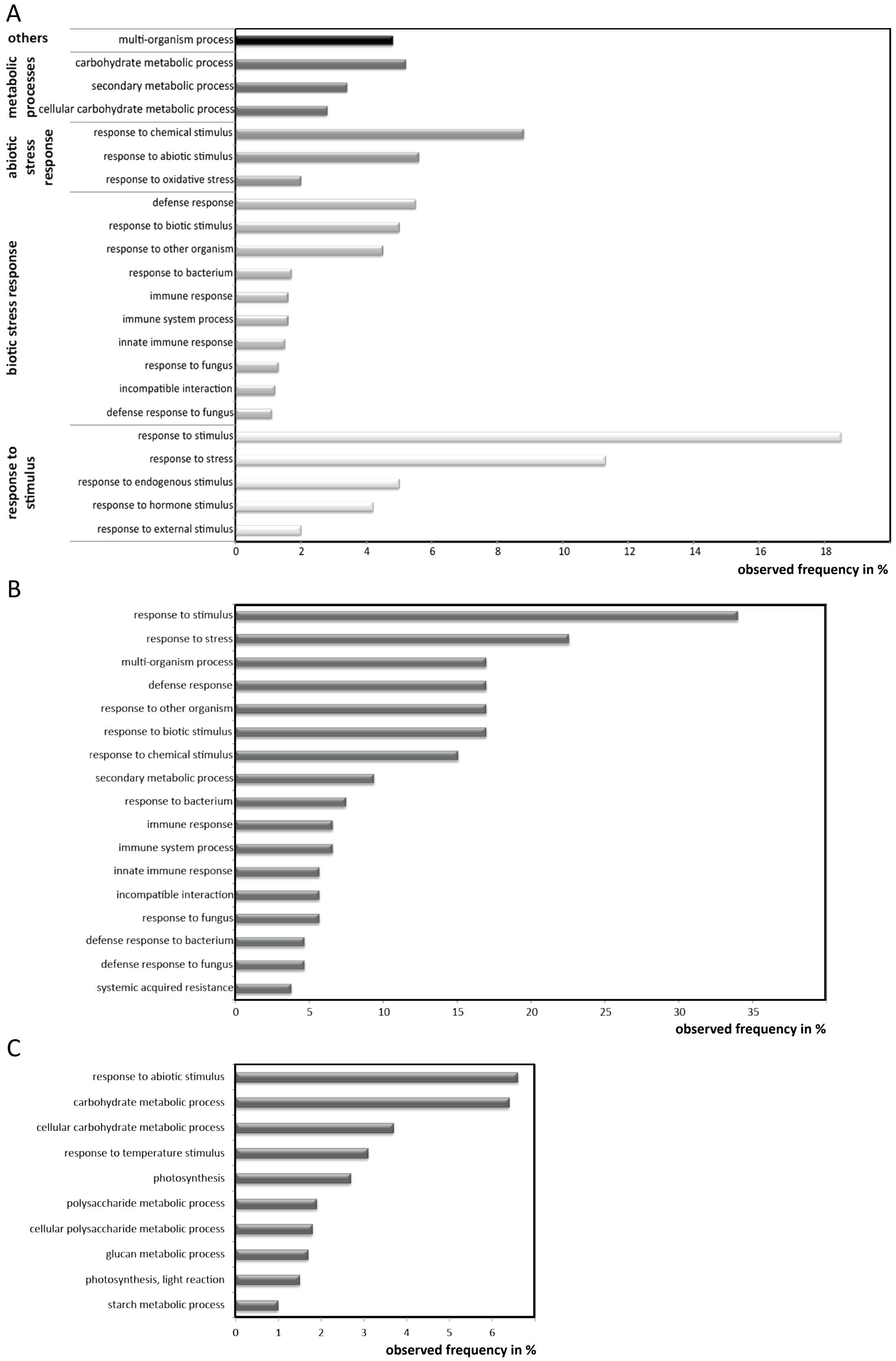

Figure 2.6: Defense-related transcriptional reprogramming in EDS1-YFP-NLS #A5 plants. (A) Gene Ontology (GO) term enrichment analysis of all differentially regulated genes with at least 2-fold changes and p-value < 0.01 in EDS1-YFP-NLS #A5 compared to Col-0. GO terms were clustered into functional groups. **(B)** GO term enrichment analysis of all at least 10 times induced genes of EDS1-YFP-NLS #A5 compared to Col-0 with a p-value < 0.05. **(C)** GO term enrichment analysis of all at least 2-fold repressed genes of EDS1-YFP-NLS #A5 compared to Col-0 with a p-value < 0.05. GO term analysis (GO biological process) was performed using the platform VirtualPlant 1.3 (Katari et al., 2010); p-values were calculated with the Fisher Exact Test (with FDR correction); p-value cutoff was set to 0.01; Arabidopsis thaliana Columbia tair10 ATH1 was used as background population.

Defense-related transcriptional regulation in #A5 is supported by comparing induced or repressed genes of #A5 with transcriptional changes in plants upon *Pst* AvrRps4 inoculation (Bartsch et al., 2006). 38 % of the induced genes in #A5 were also up-regulated in Ws-0 6h after infection with *Pst* AvrRps4 and 32 % of transcriptionally repressed genes were down-regulated in Ws-0 upon pathogen challenge as well. A hypogeometrical test against all differentially-expressed genes of the ATH1 chip revealed a high statistical significance for changes of induced and repressed genes (p-value < 0.001). Gene expression changes are illustrated by a heatmap of induced and repressed genes of both experiments (Figure 2.5B). Bartsch et al. (2006) identified 30 induced genes upon *Pst* AvrRps4 infection in an EDS1- and PAD4-dependent manner. Interestingly, 23 out of the 30 identified genes displayed also elevated transcript levels in unchallenged # A5 plants (data not shown).

Additionally, I investigated selected genes known to play a role in resistance. The strongest induced gene in #A5 was *PR1*, a prominent marker gene of SA signaling (Laird et al., 2004) (Table 2.1). Moreover, all analyzed genes involved in SA signaling were upregulated, indicating an activation of the salicylic acid (SA) signaling pathway in untreated # A5 plants. It was shown that SA and jasmonic acid (JA) pathways can act antagonistically (Pieterse et al., 2012). Therefore, I was interested in examining regulation of genes known to be involved in JA signaling. Indeed, commonly used JA marker genes, as *PDF1.2* and *VSP2*, were repressed in #A5, as well as *OPDA*, involved in JA biosynthesis. This suggests a suppression of JA signaling by the SA pathway (Table 2.1). However, the picture is not as clear since other genes of JA signaling and biosynthesis (e.g. *COI1*, *HEL*, *PR3*) showed enhanced transcript levels. Our collaborators performed a thorough analysis of genes involved in JA signaling and concluded that only few JA-responsive genes were affected, but not JA signaling network *per se* (R. Solano, personal communication). This suggests a more indirect impact on JA signaling or an effect on only particular sub-branches of the JA pathway.

Beyond genes related to SA signaling, other EDS1-dependent genes known to be differentially regulated upon pathogen challenge showed a similar trend in #A5. *FMO1* was previously described as induced upon *Pst* AvrRps4 infection as part of an SA-independent branch (Bartsch et al., 2006) and showed elevated transcript levels in #A5. Genes known to be repressed in an EDS1-dependent manner after pathogen challenge were also down-regulated in #A5 (Table 2.1). Moreover, genes involved in biosynthesis of the phytoalexin camalexin were induced, indicating an activation of this branch of defense.

Taking together, the data supports a defense-related transcriptional reprogramming in 4-week-old #A5 plants. Line EDS1-YFP-NLS #B2 showed no increase in defense gene expression and only marginal enhanced *EDS1* transcript levels compared to Col-0 (Figure 2.7).

Table 2.1: Expression of defense-related genes in EDS1-YFP-NLS #A5. List of selected defense-related genes and the fold change of their transcript levels (p-value < 0.05) in unchallenged EDS1-YFP-NLS #A5 plants compared to Col-0.

Biological process	ATG Gene ID	Gene Description	fold change EDS1-YFP-NLS #A5 vs Col-0
	AT3G48090	*EDS1*	3.30
	AT5G14930	*SAG101*	1.74
	AT3G52430	*PAD4*	7.06
SA signaling	AT2G14610	*PR1*	272.99
	AT3G57260	*PR2*	35.24
	AT5G13320	*PBS3*	26.30
	AT1G74710	*ICS1*	9.38
	AT5G26920	*CBP60g*	6.13
	AT1G64280	*NPR1*	1.32
SA-independent EDS1 pathway	AT1G19250	*FMO1*	16.40
	AT4G12720	*NUDT7*	1.61
EDS1-dependent negative regulators	AT3G46130	*MYB48*	0.68
	AT5G15410	*DND1*	0.66
	AT2G26330	*ERECTA*	0.50
JA signaling	AT3G04720	*HEL*	6.02
	AT3G12500	*PR3*	3.77
	AT3G23250	*MYB15*	3.68
	AT2G39940	*COI1*	1.79
	AT5G44420	*PDF1.2*	0.39
	AT5G24770	*VSP2*	0.14
	AT1G76680	*OPDA*	0.57
Camalexin biosynthesis	AT3G26830	*PAD3*	13.39
	AT2G30770	*CYP71A13*	10.62

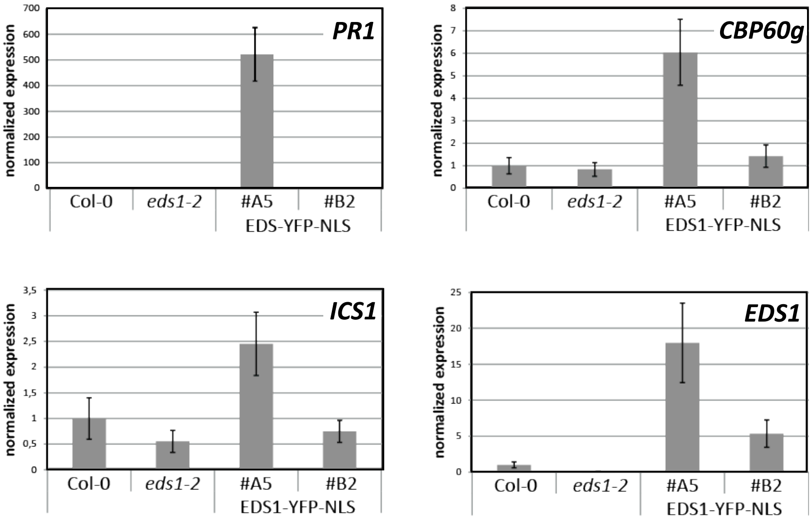

Figure 2.7: Defense gene activation in EDS1-YFP-NLS #A5 but not in #B2. Transcription levels of defense marker genes in 5-week-old EDS1-YFP-NLS #A5 plants. Expression levels were normalized using the internal control *UBIQUITIN*. Error bars represent standard deviation of 3 technical replicates. Similar results were obtained in three independent experiments.

2.2.2 SA accumulation

EDS1 regulates SA accumulation upon pathogen challenge (Wiermer et al., 2005; Vlot et al., 2009). Genes important for SA signaling were induced in 4-week-old EDS1-YFP-NLS #A5 plants (Table 2.1). This prompted me to measure SA levels in healthy #A5 and #B2 plants.

Free and total SA levels were higher in #A5 plants compared to Col-0, *eds1-2* and EDS1-YFP, consistent with an activation of the SA defense pathway (Figure 2.8). SA measurements in #A5 were rather variable. A possible reason might be the differences in developmental stages of single plants. I observed variations in the severity of growth defects in #A5 plants dependent on the specific light and humidity conditions for single pots within trays of plants grown in climate chambers. For future SA analysis, plants can be shifted to 19°C to synchronously trigger #A5 outputs and thereby obtain a more uniform phenotype (see section 2.3). Line #B2, with low EDS1 expression, accumulated similar free and total SA levels as Col-0 (Figure 2.8). This is consistent with the finding that #B2 also showed no growth defects and no auto-induced defense gene expression, suggesting that induction of defense responses are not activated in #B2.

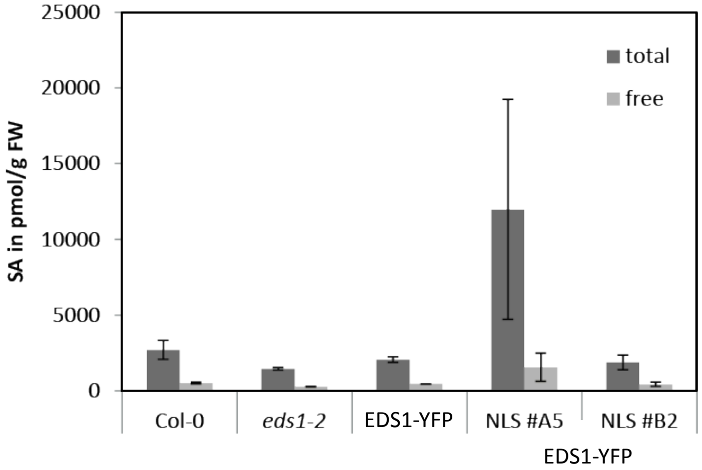

Figure 2.8: Elevated SA accumulation in EDS1-YFP-NLS #A5. Total and free SA levels was determined in leaf tissue of 5-week-old plants of the depicted genotypes. Error bars represent standard deviation of three technical replicates. Similar results were obtained in three independent experiments.

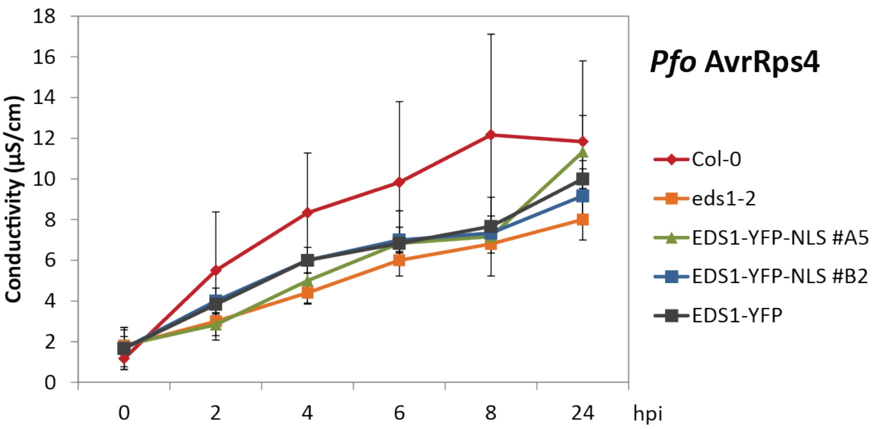

Figure 2.9: Cell death response upon avirulent pathogen challenge. Five-week-old Col-0 plants were infiltrated with *Pfo* expressing AvrRps4 and conductivity was measured at the indicated time points. Error bars represent standard deviation of 6 technical replicates. Similar results were obtained in two independent experiments.

2.2.3 Cell death induction

One morphological characteristic of EDS1-YFP-NLS plants with high EDS1 accumulation the formation of necrotic lesions (see section 2.1). Plants are able to execute localized programmed cell death to prevent pathogens growth. I was interested in analyzing if the observed cell death in EDS1-YFP-NLS #A5 is defense-related. Therefore, EDS1-YFP-NLS #A5 plants were examined for spontaneous cell death as a further readout of induced defense activity. Additionally, I monitored their capability to develop hypersensitive response (HR), a form of defense-related programmed cell death upon pathogen effector trigger.

Ion leakage as a proxy to cell death was monitored in pathogen-challenged plants. For this, Arabidopsis leaves were syringe-infiltrated with the bacterial strain *Pseudomonas fluorescens* (*Pfo*), a nonpathogen of Arabidopsis which does not cause disease symptoms. After genetic modification, this strain is able to secrete effectors via the type III secretion apparatus into plants cells (van Dijk et al., 2002; Guo et al., 2009). A *Pfo* strain expressing the effector AvrRps4, recognized by RPS4 (Hinsch and Staskawicz, 1996), was used to induce cell death. Cell death response in Col-0 to AvrRps4 is very weak; no significant difference were detected in Col-0 compared to *eds1-2* negative control. All further tested lines, including #A5, exhibited ion leakage in a similar range (Figure 2.9). However, based on this experiment it can be concluded that #A5 plants do not induce massive effector-triggered cell death.

In another approach, cell death was visualized by trypan blue staining. At first, I analyzed spontaneous cell death in 5-week-old plants grown at 22°C. No staining of dead cells could be observed in Col-0, *eds1-2*, EDS1-YFP, #A5 or #B2 (data not shown). Line #A5 defense outputs can be amplified at lower temperatures (see section 2.3). Hence, cell death was determined after plants were shifted for one week to 19°C. Indeed, temperature shift triggered punctuate cell death lesions in #A5 which were not observed in Col-0 (Figure 2.10A). However, #A5 plants showed yellowish leaves developing into necrosis in aging plants (see section 2.1). These changes occurred before senescence symptoms start in wt plants. Therefore, it is hard to distinguish if cell death in line #A5 in response to low temperature is a specific defense-related feature or due to an accelerated aging processes.

Pathogen-induced HR was investigated upon inoculation with the avirulent *H. arabidopsidis* isolate Emwa1. Cells were stained at 6 dpi. In Col-0, defined HR lesions were detected (Figure 2.10B). A similar pattern was observed in EDS1-YFP and #B2 lines. In contrast, *eds1-2* plants were fully susceptible, illustrated by expanded hyphae growth. However, line #A5 showed spreading cell death associated with potentially aborted hyphae growth. Notably, a higher number of putative infection sites were detected in #A5 compared to Col-0, suggesting a lower threshold for initiation and spread of cell death upon pathogen recognition. It is important to note that after *H. arabidopsidis* inoculation, plants were shifted to 19°C to promote a successful propagation of the pathogen. As described above, #A5 plants transferred to lower temperature showed spontaneous cell death. Hence, it is difficult to separate pathogen- from temperature-induced cell death in this experimental setup. It is possible that the increased amount of putative infection sites in #A5, defined by dead plant cells, is not caused by pathogen recognition but due to the effect of low temperature. An approach to determine if the HR-like spots were pathogen-induced might be by examining their association with aborted hyphae growth and thereby distinguish them from temperature-triggered cell death. Another assay to separate cell death in response to pathogens from temperature effects will be by repeating the experiment without shifting plants to 19°C upon inoculation. For this, it has to be assured that *H. arabidopsidis* still propagates normally at higher temperature in the control lines Col-0 and *eds1-2*.

Taking together, #A5 exhibited accelerated cell death. It remains unclear whether the observed cell death is defense-related or a characteristic of its growth and development defects. Remarkably, line #B2 showed wt-like TIR-NB-LRR-mediated programmed cell death. This result provides evidence that nuclear EDS1 is at least sufficient for HR initiation in RPS4-triggered resistance.

In summary, high nuclear-restricted EDS1 accumulation induces defense responses such as defense-related transcriptional reprogramming, enhanced SA accumulation and spontaneous cell death in unchallenged plants. These features together with the observed growth inhibition are inherent to plants expressing constitutive resistance response, suggesting autoimmunity of #A5. Autoimmune-mutants usually show enhanced disease resistance (Bowling et al., 1994, 1997; Yoshioka et al., 2001; Li et al., 2001; Shirano et al., 2002; Yang and Hua, 2004). Resistance phenotypes of #A5 plants are further analyzed in section 2.5. By contrast, plants with lower amounts of nuclear EDS1 (line #B2) showed wt-like behavior in all tested assays. I conclude that high EDS1 amounts restricted to the nucleus activate defense responses in plants without a pathogen effector trigger. This correlates with the observed growth defects only the selected EDS1-YFP-NLS lines with strong EDS1 accumulation. Additionally, it was demonstrated that nuclear EDS1 is sufficient to induce cell death.

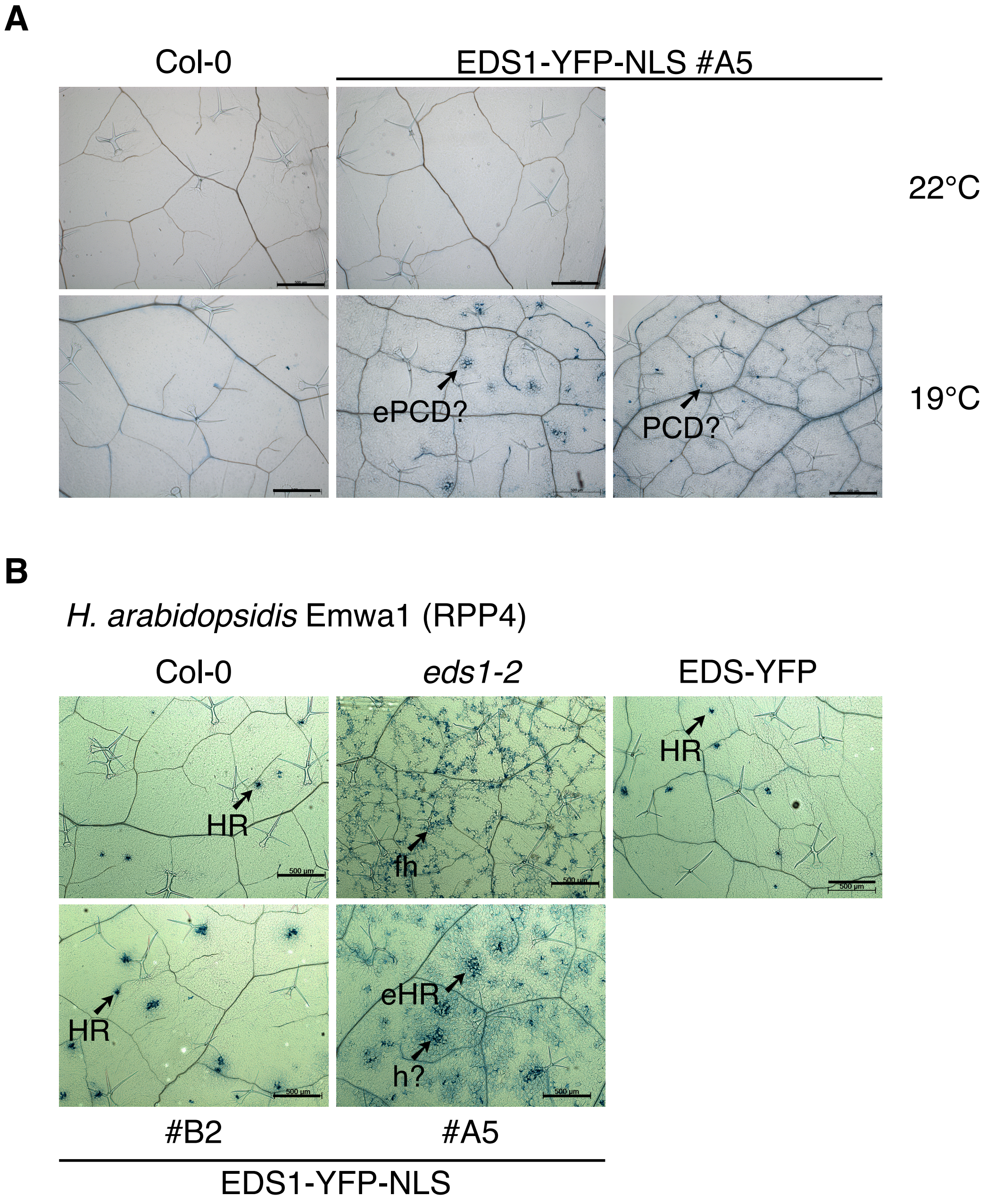

Figure 2.10: Extended cell death in EDS1-YFP-NLS #A5 plants after temperature shift to 19°C and inoculation with avirulent pathogens. Visualization of dead cells and pathogen growth in leaves through staining with lactophenol blue. **(A)** Five-week-old plants grown at 22°C (top) and after shift for one week to 19°C (bottom). **(B)** Five-week-old plants of the indicated genotypes were inoculated with *H. arabidopsidis* Emwa1 and stained 6 dpi. PCD: programmed cell death; ePCS: extended PCD; HR: hypersensitive response associated cell death; fh: free pathogen hyphae; h?: putative aborted hyphae growth. Bar = 500μm.

2.3 Temperature dependency of EDS1-YFP-NLS phenotypes

It is a common feature of plants with constitutive activated defense responses to show dwarfism. This form of growth inhibition is often relieved when plants are grown at higher temperature (Yang and Hua, 2004; Ichimura et al., 2006). Therefore, I tested whether this characteristic of autoimmunity is shared by EDS1-YFP-NLS plants which displayed growth defects. Indeed, when EDS1-YFP-NLS #A3 and #A5 plants were grown at 28°C, growth inhibition was relieved (Figure 2.11). Differences between the lines also disappeared at the molecular level at higher temperatures. The #A5 plants grown at 28°C displayed similar EDS1 accumulation as Col-0 grown at 22°C, whereas #A5 grown at 22°C displayed elevated EDS1 levels (Figure 2.12). This suggests a correlation between increasing amounts of EDS1 and stunted growth, mentioned above (see section 2.1). Previous work by Yang and Hua (2004) described a reduction of *EDS1* gene expression at higher temperature. A decrease of *EDS1* transcript levels in Col-0 grown at 28°C compared to plants grown at 22°C was observed in this study (Yang and Hua, 2004), indicating that *EDS1* gene expression is reduced at higher temperature leading to lower EDS1 protein accumulation. Temperature had no strong impact on protein accumulation in EDS1-YFP plants, as demonstrated by elevated levels at both temperatures (Figure 2.12). A possible explanation for temperature-independent EDS1 accumulation in EDS1-YFP might be a positioning effect. Even though it is driven by the native promoter like the *EDS1-YFP-NLS* transgene, insertion site of the transgene inside the genome can influence gene expression (Hobbs et al., 1990; Weiler and Wakimoto, 1995). Enhanced EDS1-YFP-NLS #A5 protein levels at 22°C correlate with increased gene expression of *PR1* and *ICS1* (Figure 2.13), re-emphasizing the connection between high enforced nuclear EDS1 and defense gene induction. The growth defects of #A5 were reversible. When #A5 plants already exhibiting stunted growth were transferred to 28°C, they became indistinguishable from Col-0 two weeks after shift (Figure 2.14). Also, when #A5 plants were grown at 28°C and shifted to 19°C, they started to show developmental defects accompanied by defense phenotypes such as EDS1 accumulation and defense gene induction (see section 2.9.2). The strength of the developmental phenotype was accelerated by shifting plants to 19°C (Figure 2.14). This feature can be exploited to accelerate and amplify defense-like outputs of #A5.

In summary, growth defects of EDS1-YFP-NLS #A5 plants were suppressed at higher temperature, correlating with a reduction of EDS1 protein levels and inhibition of defense gene expression.

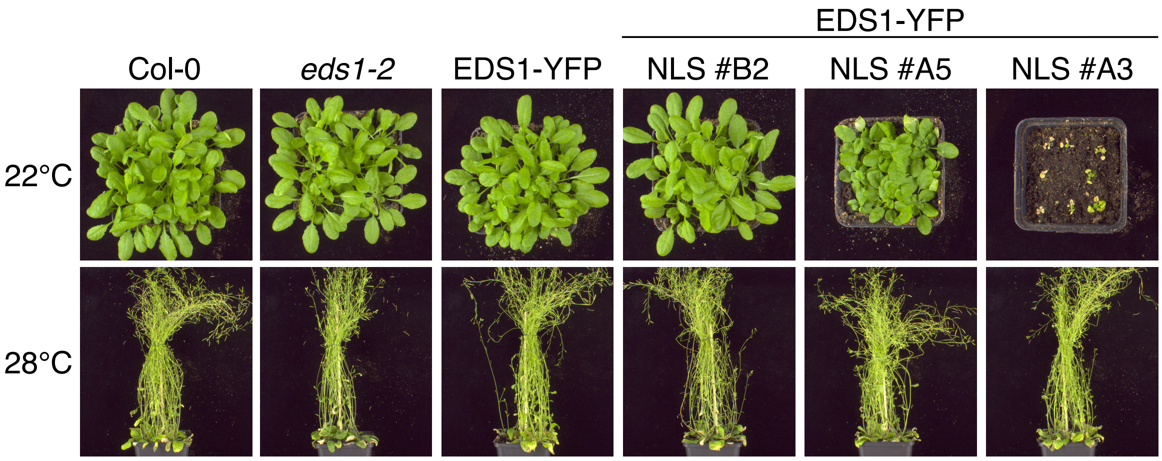

Figure 2.11: Growth defects of EDS1-YFP-NLS lines are relieved when plants are grown at higher temperature. Plants of the depicted genotypes were grown at either 22°C (top) or 28°C (bottom) for six weeks.

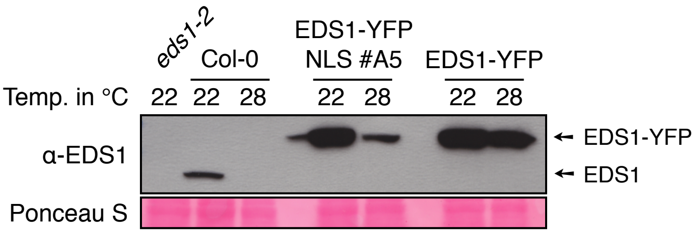

Figure 2.12: Reduced EDS1 protein accumulation of EDS1-YFP-NLS #A5 plants grown at high temperature. Immunoblot analysis showing expression of EDS1 in 4-week-old plants grown at 22°C or 28°C. The membrane was probed with α-EDS1 antibody. Ponceau S staining of the membrane indicates equal loading and transfer onto the membrane. Migration of wt EDS1 and EDS1-YFP is marked by arrows on the right.

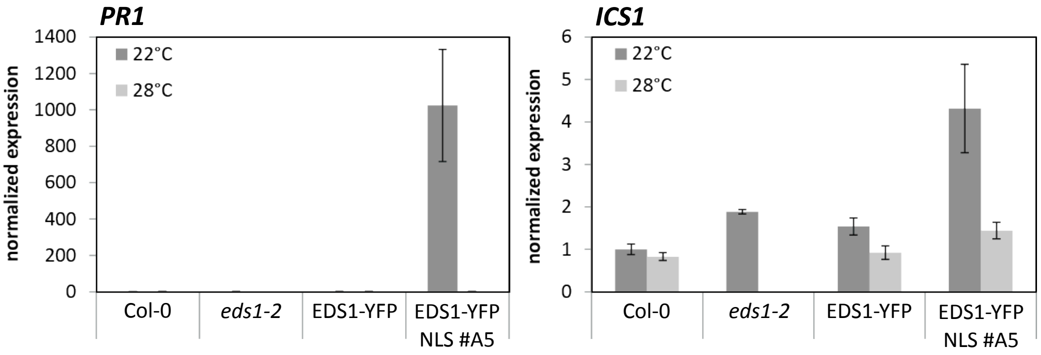

Figure 2.13: Expression of defense marker genes in EDS1-YFP-NLS #A5 plants is repressed at high temperature. Leaf samples of plants of the indicated genotypes were grown for 4 weeks at either 22°C or 28°C. Transcript levels were determined and normalized using the internal control *UBIQUITIN*. Error bars represent standard deviation of three technical replicates.

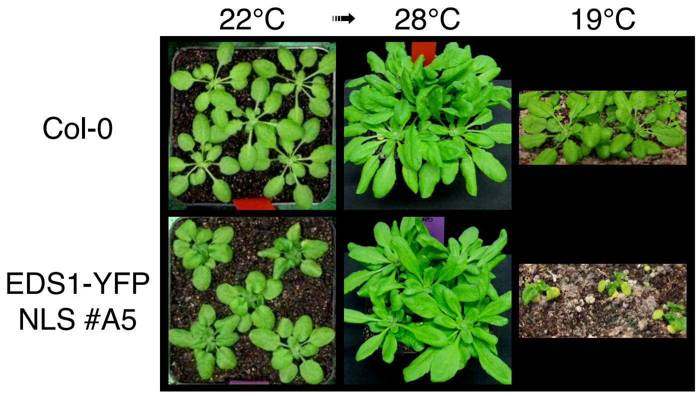

Figure 2.14: Growth defects of EDS1-YFP-NLS #A5 can be reversed by shifting plants to higher temperatures and are accelerated when plants are grown at lower temperatures. Col-0 and EDS1-YFP-NLS #A5 plants were grown for 4 weeks at 22°C (left panel) and then transferred for 2 weeks to 28°C (middle panel). Plants shown at the right panel were grown for four weeks at 19°C.

2.4 EDS1-YFP-NLS phenotypes in aging plants

The observed growth defects of EDS1-YFP-NLS #A5 were increased in older plants (Figure 2.3). Therefore, I monitored the development of EDS1-YFP-NLS #A5 plants at the molecular level in more detail. It is known that *EDS1* transcripts accumulate in aging plants (Figure 2.15A). There is very low expression in germinated seeds but it increases during seedling development. Expression reaches its highest level in young rosette leaves and maintains high until bolting stage. Additionally, strong *EDS1* levels are present in young flowers, whereas in senescing leaves, *EDS1* transcript levels are low. Given that #A5 plants express the EDS1-YFP-NLS construct under control of the native *EDS1* promoter, a similar pattern of transgene transcript level might be expected. Leaf tissue samples were taken of 3-, 4-, 5- and 6-week-old plants to analyze gene expression and protein accumulation. By determining *EDS1* transcript levels, a slight trend of transcript accumulation over time was observed in #A5 (Figure 2.15B). This trend could not be detected in Col-0 or any other tested line, inconsistent with the expected increase in wt plants mentioned above. However, #A3 plants, which showed the strongest growth defects, had extremely high *EDS1* transcript expression in 3-week-old plants. This observation suggests a correlation between severity of developmental defects and *EDS1* accumulation.

Furthermore, I investigated EDS1 protein amounts at different stages of development. There was a clear increase of EDS1-YFP-NLS #A5 protein in rosette leaves from week 3 to week 6 (Figure 2.16). EDS1 levels in Col-0 did not significantly increase over time. In line #B2, EDS1 protein is hardly detectable except in the 4-week sample. It is considered as an experimental error because nearly absent EDS1 levels one week after strong accumulation, whereas all other lines had increasing or at least stable EDS1 amounts, are highly unlikely. In line #A3, EDS1 levels were only monitored in 3-week-old plants since they are dying shortly afterwards. Massive protein accumulation, higher than in all other measured samples, were monitored. This correlates with the strongest growth defects displayed by #A3 plants. As

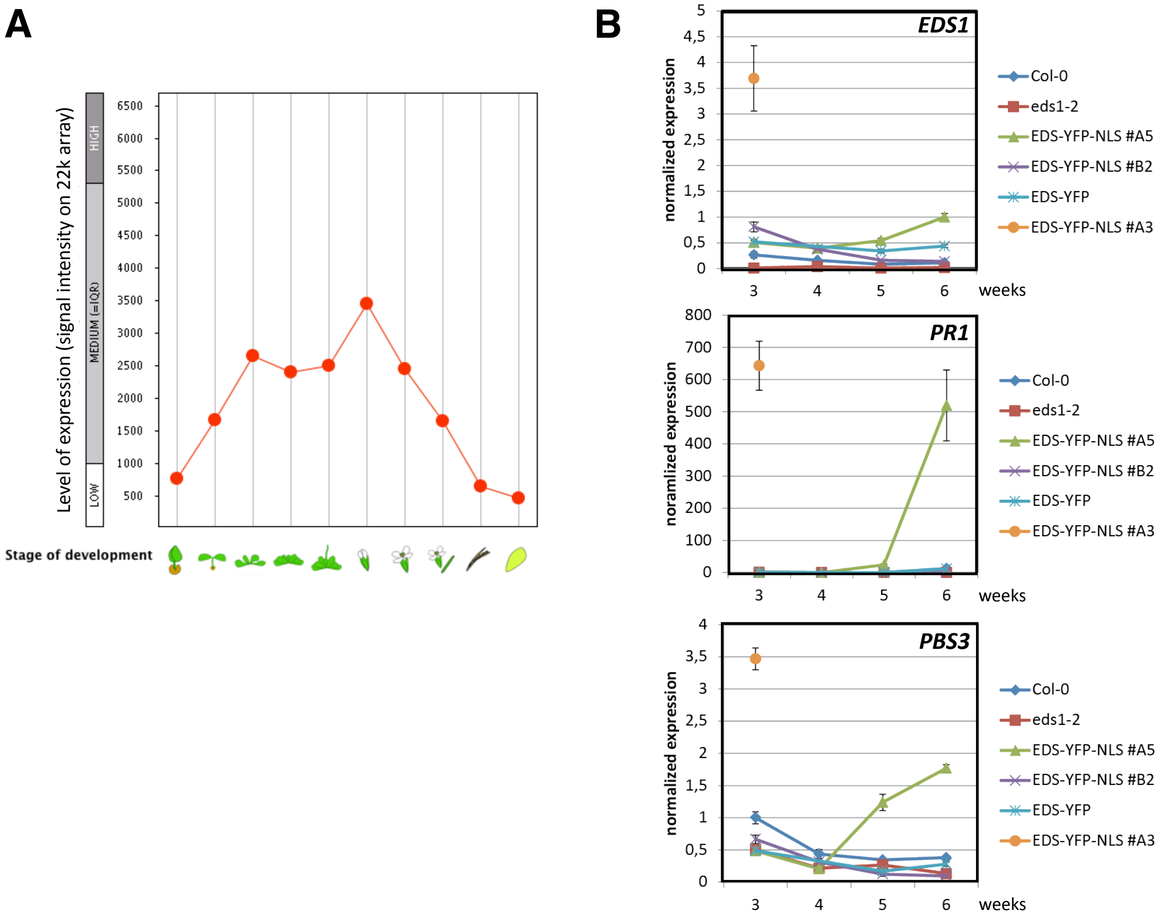

Figure 2.15: *EDS1* gene expression during plant development. (A) *EDS1* transcript accumulation in plants based on public available microarray data using the genevestigator platform (Hruz et al., 2008). **(B)** *EDS1* transcript levels were monitored in 3-week to 6-week-old plants of the depicted genotypes. Leaf samples were collected at the indicated time points. Transcriptional expression was normalized using the internal control *UBIQUITIN*. Error bars represent standard deviation of three technical replicates. Similar results were obtained in two independent experiments.

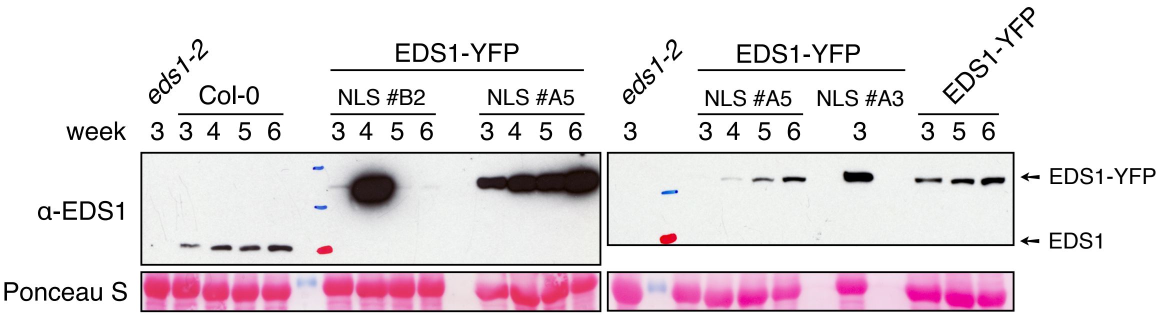

Figure 2.16: Increase of EDS1 protein levels in EDS1-YFP-NLS #A5 plants over time. Immunoblot analysis was performed on total leaf extracts of the depicted genotypes at the indicated time points. The membrane was probed with α-EDS1 antibody. Ponceau S staining was performed to ensure equal loading. wt EDS1 protein and EDS1-YFP are labeled on the right.

described above, EDS1-YFP plants express marginal higher EDS1 protein levels than #A5. Despite the fact that *EDS1-YFP* is driven by the native *EDS1* promoter, parameters such as developmental stage or temperature, usually influencing EDS1 protein expression, had no strong impact on this line (see section 2.3). Attainment of a potential peak-accumulation of EDS1 in plants cannot be an explanation for the observed effects, since #A3 proves that even higher levels are possible. A reason might be different insertion sites of the constructs, leading to constitutively high expression, as mentioned above (see section 2.3). Strong protein accumulation in EDS1-YFP is not reflected at the transcript level (Figure 2.15B), indicating that post-transcriptional mechanisms might be the reason for high EDS1 levels. These observations support the initial hypothesis of a correlation between EDS1 protein accumulation and developmental defects in EDS1-YFP-NLS plants. Increasing levels of EDS1-YFP-NLS were not reflected to the same extent on the transcript level, suggesting that EDS1 activity is controlled by protein stabilization.

If growth defects in line #A5 are connected to defense response, I would assume amplification of the described defense outputs in aging plants. Indeed, transcriptional upregulation over time of defense marker genes such as *PR1* and *PBS3* were monitored. Accordingly, line #A3 showed very high transcript levels of both genes (Figure 2.15B). Moreover, SA accumulation was measured in 4- and 5-week-old plants. Already elevated levels observed in 4-week-old #A5 plants compared to Col-0, *eds1-2* and #B2 increased after 5 weeks (Figure 2.17). Taking together, this data suggests that an increase of EDS1 protein levels in aging #A5 plants correlates with growth defects, which are accompanied by amplification of defense outputs.

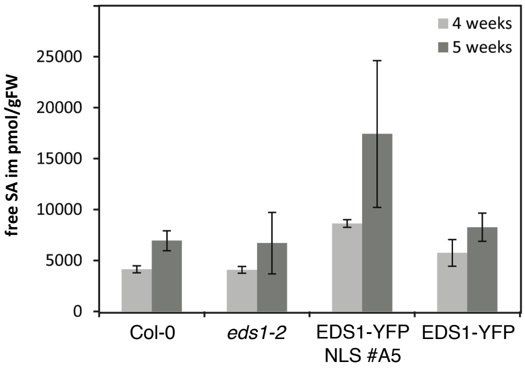

Figure 2.17: SA accumulation in EDS1-YFP-NLS #A5 plants is age-dependent. Free SA levels were determined in leaf tissue of 4- and 5-week-old plants of the depicted genotypes. Error bars represent standard deviation of three technical replicates.

2.5 Basal and *R* gene-mediated resistance in EDS1-YFP-NLS

Enhanced SA accumulation and defense gene induction in EDS1-YFP-NLS #A3 and #A5 indicate a constitutive resistance response. Therefore, a reasonable next step was to analyze the resistance phenotype of EDS1-YFP-NLS plants. Given that line #A3 dies early after germination, lines #A5 (high EDS1 level) and #B2 (low EDS1 level) were included in the analysis. If not stated otherwise, 5-week-old plants were used for infection assays. At that stage, #A5 plants were beginning to show developmental changes but still retained wt-like leaf shape to exclude the possibility that morphological abnormalities influence the experiment.

The TIR-NB-LRR protein RPS4 needs to localize to the nucleus to confer resistance to *Pseudomonas* strains carrying the effector AvrRps4 and RPS4-triggered resistance requires EDS1 (Wirthmueller et al., 2007). García et al. (2010) demonstrated a nuclear enrichment of EDS1 within the first 1 to 3 hours during RPS4-mediated resistance, consistent with a critical role of nuclear EDS1 during defense activation. I hypothesize that EDS1-YFP-NLS #A5 is in an active state with enhanced capacity to resist pathogen attack. Hence, I studied the resistance response of #A5 and #B2 by spray inoculation of 5-week-old plants towards the avirulent *Pseudomonas syringae* pv. *tomato* (*Pst*) strain DC3000 expressing the effector AvrRps4 (hereafter referred to as *Pst* DC3000 AvrRps4). First, EDS1 protein levels upon pathogen challenge was monitored. An increase of EDS1 occurred in #A5 24h after spray inoculation with *Pst* DC3000 AvrRps4 (Figure 2.18). Thus, #A5 maintained the ability to increase EDS1 levels upon pathogen infection. The control line EDS1-YFP line complemented wt-like resistance in all tested pathogen assays (Figure 2.19A; García et al. (2010)). Also, lines #A5 and #B2 displayed wt-like resistance. To test if plants used for pathogen assays indeed exhibited induced defense outputs before infection, I measured transcript levels of defense marker genes and could verify their induction. Additionally, I investigated resistance response to *Pst* DC3000 AvrRps4 in older plants, when #A5 already exhibited growth abnormalities. Resistance was intact but not enhanced in #A5 compared to wt, irrespective of the age (data not shown). I concluded that the resistance phenotype of

#A5 to avirulent pathogens is independent of its developmental stage. Col-0 is resistant to *Pst* DC3000 AvrRps4. I reasoned if EDS1-YFP-NLS plants express enhanced resistance, it might be more obvious by analyzing basal immunity. Therefore, susceptibility was tested in response to two virulent plant pathogens; the bacterial strain *Pst* DC3000 and the oomycete *Hyaloperonospora arabidopsidis* (*H. arabidopsidis*) isolate Noco2 (Figure 2.19B and C). Bacterial titers were measured 3 days after spray inoculation with *Pst* DC3000. No statistical significant differences were detected between Col-0, EDS1-YFP and the EDS1-YFP-NLS lines #A5 and #B2 in each single experiment. However, there is a reproducible trend of enhanced resistance in #A5. When the data of all performed *Pst* DC3000 infections were combined, the trend of enhanced resistance of #A5 is statistically significant (Table 2.2). Additionally, upon *H. arabidopsidis* infection, a similar minor reduction in susceptibility of #A5 compared to Col-0 was observed (Figure 2.19C).

Interestingly, line #B2, which expresses less nuclear EDS1 protein compared to Col-0 (Figure 2.4), was not compromised in the tested resistance responses. This result provides evidence that even small amounts of nuclear EDS1 are sufficient for basal and RPS4-mediated resistance.

Taking together, this data suggests that defense outputs induced by high nuclear-restricted EDS1 result in mildly enhanced resistance to virulent pathogens. Previous studies showed an intermediate level of basal and *R* gene-mediated resistance in transgenic lines in which EDS1 is excluded from the nucleus (García et al., 2010). Based on this result, the authors hypothesized that cytosolic and nuclear EDS1 is required to achieve resistance. However, the data presented here lead me to conclude that nuclear EDS1 is sufficient to trigger wt-like resistance under the analyzed conditions.

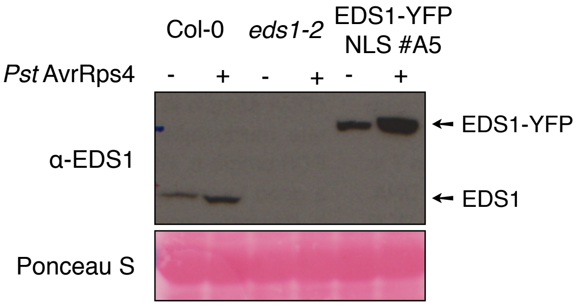

Figure 2.18: Induced EDS1 protein accumulation upon avirulent pathogen challenge. Four-week-old plants were spray inoculated with *Pst* DC3000 AvrRps4 and leaf samples harvested before and 24h after pathogen treatment. Immunoblot analysis was performed on total protein extracts and the membrane was probed with α-EDS1 antibody. Ponceau S staining of the membrane indicates equal loading and transfer onto the membrane. wt EDS1 protein and EDS1-YFP are labeled on the right.

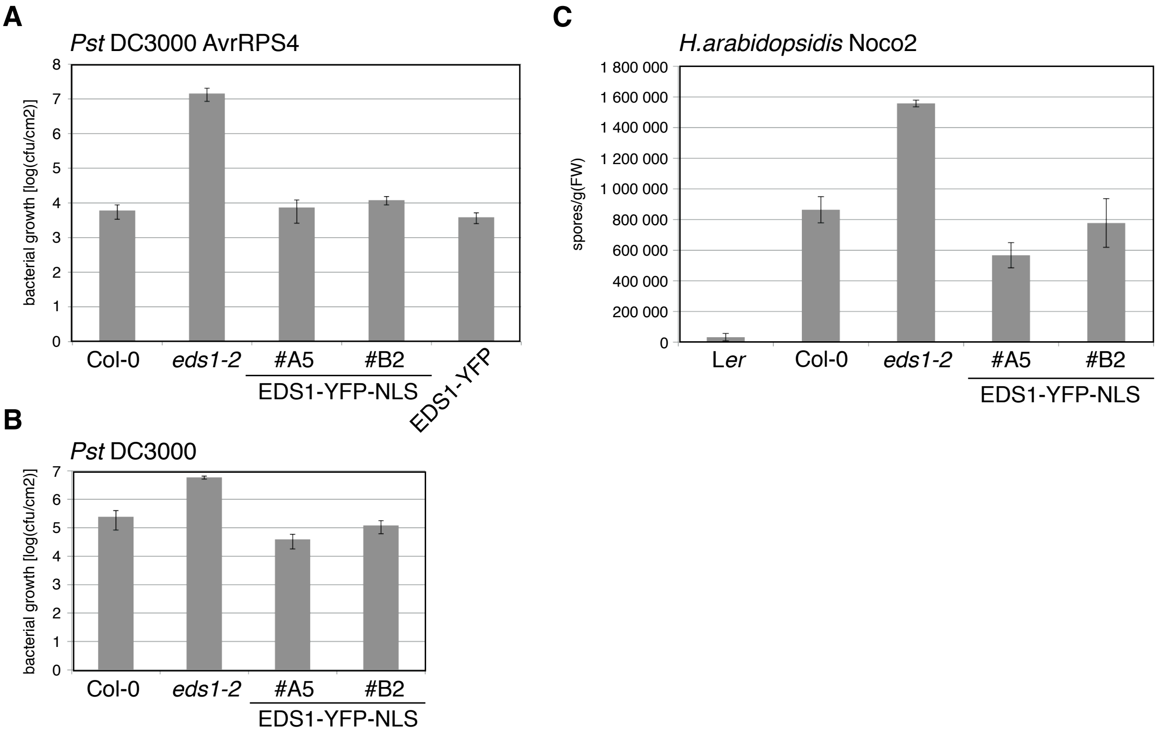

Figure 2.19: Basal and RPS4-mediated resistance in EDS1-YFP-NLS lines. (A) Five-week-old plants of the depicted genotypes were spray-infected with the avirulent bacterial strain *Pst* DC3000 AvrRps4. Bacterial titers were measured at 3 dpi. Error bars represent standard error of 5 technical replicates. The experiment was repeated at least 3 times with similar results. **(B)** Five-week-old plants of the depicted genotypes were spray-infected with virulent *Pst* DC3000. Bacterial titers were measured at 3 dpi. Error bars represent standard error of 5 technical replicates. The experiment was repeated at least 3 times with similar results. **(C)** Pathogen conidiospores were counted of leaves 7 dpi with virulent *H. arabidopsidis* Noco2. Values are the average of 4 replicate samples and error bars represent standard deviation of the mean. Similar results were obtained in at least three independent experiments.

Table 2.2: Statistically significant enhanced resistance of EDS1-YFP-NLS #A5 plants upon pathogen challenge with the virulent bacterial strain *Pst* DC3000. Anova test was performed of 3 independent experiments. Differences between the experiments is higher than between genotypes. Df: Degrees of freedom; Sq: square.

	Df	Sum Sq	Mean Sq	F-value	p-value
experiment	2	313.39	156.70	55.90	< 0.005
genotype	1	24.91	24.91	8.89	0.0069
residuals	22	61.67	2.80		

2.6 Impact of the SA pathway on EDS1-YFP-NLS phenotypes

An important plant defense response upon infection with biotrophic pathogens is activation of the SA signaling pathway. EDS1 was shown to control SA accumulation during TIR-NB-LRR-mediated defense (Feys et al., 2001). This prompted me to test if SA biosynthesis or signaling influences EDS1-YFP-NLS #A5 defense-related outputs. Therefore, components important for the SA pathway were removed from the #A5 background by crossing with knock-out lines of these genes.

2.6.1 Influence of SA biosynthesis

Sid2-1 plants carry a mutation in *ICS1* (single base-pair mutation, produces stop codon and disrupts highly conserved chorismate-binding domain). *ICS1* encodes the major protein for SA biosynthesis in response to pathogens (Wildermuth et al., 2001a). These mutants fail to induce SA signaling such as induction of *PR* gene expression and display enhanced susceptibility (Nawrath and Métraux, 1999).

#A5/*sid2-1* plants exhibited growth defects similar to #A5 parent plants (Figure 2.20A). However, I noticed some differences by comparing development of both lines. Growth defects in #A5 were more pronounced; manifested by earlier and stronger yellowish leaves and lesion formation in addition to enhanced dwarfism. Defects in #A5/*sid2-1* appeared later and were less severe, suggesting that decreased SA accumulation delays or reduces defense outputs of #A5. Nuclear localization of EDS1-YFP-NLS #A5 was unaffected by the loss of *ICS1* (Figure 2.20B). To confirm that elevated SA levels in #A5 are caused by *ICS1*-dependent SA generation and not by alternative SA production through the phenylalanine ammonia lyase pathway (Mauch-Mani and Slusarenko, 1996), SA accumulation was measured in 5-week-old plants. #A5/*sid2-1* plants did not have enhanced SA levels but similar amounts as Col-0 and *sid2-1* mutants, indicating that SA accumulating in #A5 is indeed produced via *ICS1* (Figure 2.21). Moreover, *PR1* gene induction was abolished in #A5/*sid2-1* compared to #A5 (Figure 2.22). This was expected from the fact that SA accumulation induces *PR* gene expression (Nawrath and Métraux, 1999). These results suggest that major aspects of the #A5 phenotype are independent of the SA signaling pathway. Previous studies revealed SA-independent branches of EDS1 signaling (Bartsch et al., 2006). Activation of these SA-independent responses might induce defense outputs in #A5 plants lacking *sid2-1*. Transcriptomic analysis of #A5 compared to Col-0 revealed an increase of *FMO1* transcript levels in #A5 (Table 2.1). *FMO1* is an *EDS1*-dependent gene that activates SA-independent defense upon pathogen challenge (Bartsch et al., 2006). It will be interesting to investigate the resistance of #A5/*sid2-1* towards biotrophic pathogens in subsequent analysis.

SA contributes to a positive feedback loop which potentiates EDS1/PAD4 signaling (Vlot et al., 2009). Therefore, EDS1 accumulation in #A5/*sid2-1* and #A5 was analyzed. A reduced amount of the EDS1-YFP-NLS was detected in line #A5/*sid2-1* compared to #A5 (Figure 2.23). Intriguingly, there was also less wt EDS1 monitored in *sid2-1* compared to Col-0. This suggests that SA accumulation via *ICS1* has a significant impact on EDS1 levels even in healthy plants. One possible explanation for this result is, by losing the positive feedback loop, PAD4 protein level might be reduced. PAD4, in turn, is needed to stabilize EDS1 and this might result in lower EDS1 accumulation.

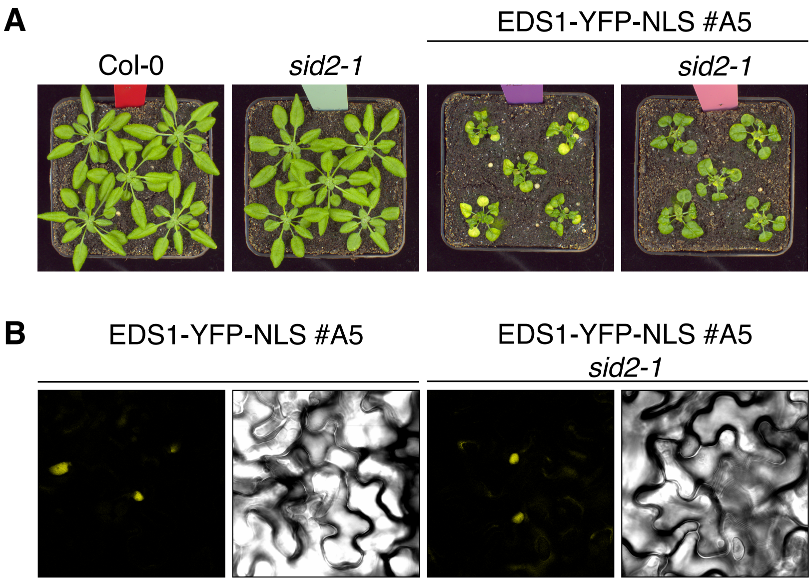

Figure 2.20: Loss of *ICS1* has no impact on growth defects displayed by EDS1-YFP-NLS #A5 and does not change subcellular distribution of EDS1-YFP-NLS protein. (A) Four-week-old plants grown at 22°C of the depicted genotypes were shifted for one week to 19°C to amplify growth defects before pictures were taken. **(B)** Confocal images of YFP fluorescence and bright field images of leaf epidermal tissue. Images were taken of 3-week-old soil grown plants.

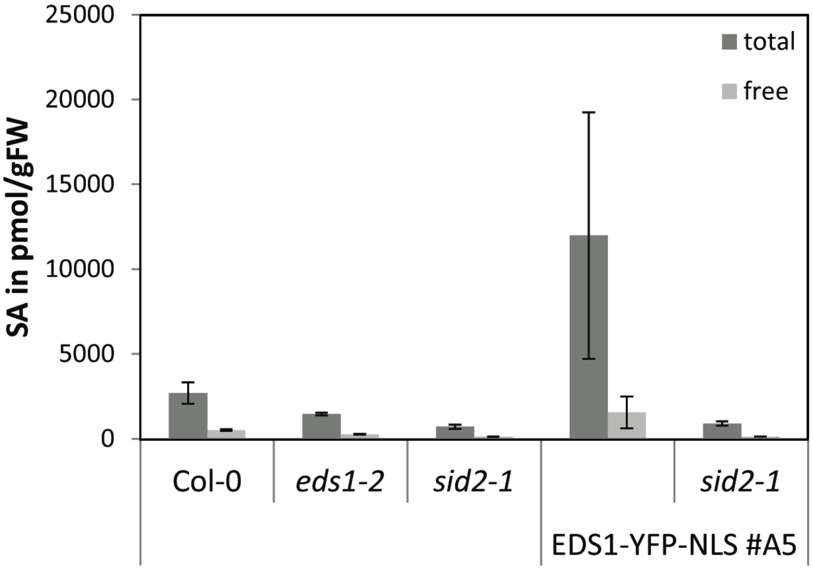

Figure 2.21: Elevated SA levels in EDS1-YFP-NLS #A5 plants are inhibited in the *sid2-1* background. Free and total SA accumulation was determined in leaf tissue of 5-week-old plants of the depicted genotypes. Error bars represent standard deviation of 4 technical replicates.

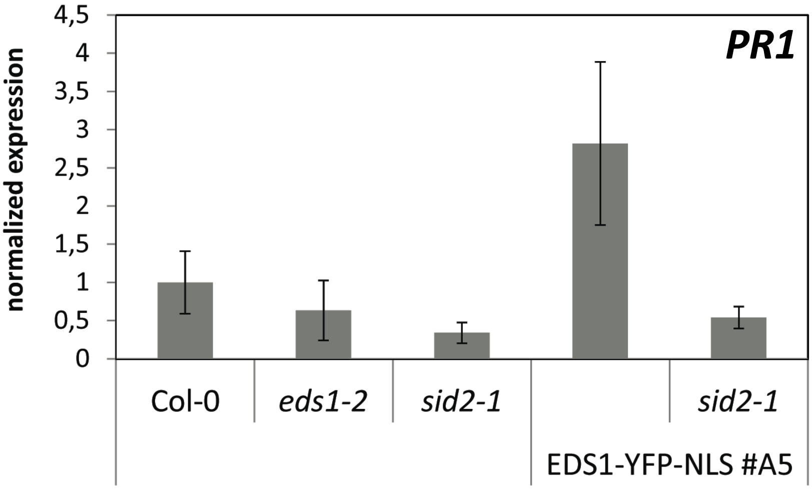

Figure 2.22: Loss of *ICS1* blocks increased *PR1* expression in EDS1-YFP-NLS #A5 plants. *PR1* transcript levels were determined in leaf samples of 5-week-old plants and normalized using the internal control *UBIQUITIN*. Error bars represent standard deviation of 3 technical replicates. Similar results were obtained in two independent experiments.

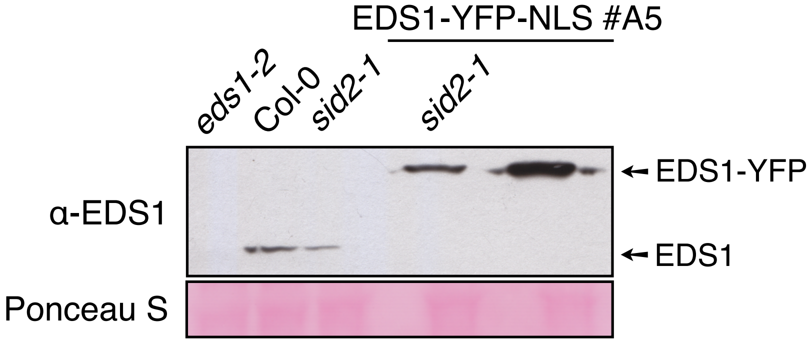

Figure 2.23: Reduced EDS1-YFP-NLS protein accumulation in plants lacking *ICS1*. Immunoblot analysis was performed on total protein extracts of 5-week-old plants. The membrane was probed with α-EDS1 antibody. Ponceau S staining of the membrane indicates equal loading and transfer onto the membrane. wt EDS1 protein and EDS1-YFP are labeled on the right.

2.6.2 Influence of SA signaling

Pathogen-induced increases in SA levels lead to transcriptional reprogramming mediated by the transcription coactivator NPR1 (Cao et al., 1994; Delaney et al., 1995; Wang et al., 2006). Removing this important component of SA signaling allows to further dissect the impact of SA signaling downstream of *ICS1* on EDS1-YFP-NLS #A5 defense outputs.

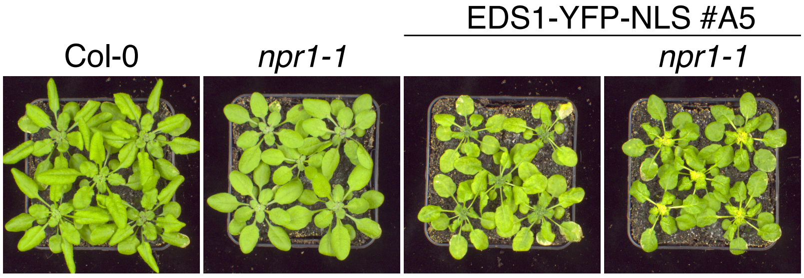

Figure 2.24: Loss of *NPR1* has no impact on growth defects displayed by EDS1-YFP-NLS #A5 plants. Four-week-old plants grown at 22°C of the depicted genotypes were shifted for one week to 19°C to amplify growth defects before pictures were taken.

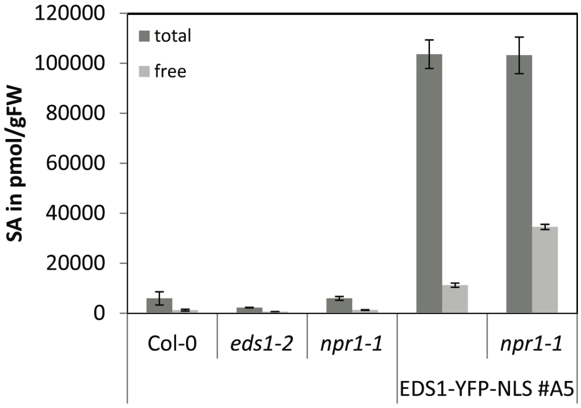

Figure 2.25: Loss of *NPR1* has no impact on elevated SA levels in EDS1-YFP-NLS #A5 plants. Free and total SA accumulation was determined in leaf tissue of 5-week-old plants of the depicted genotypes. Plants were shifted for one week to 19°C to amplify EDS1-YFP-NLS #A5 defense phenotypes. Error bars represent standard deviation of 3 technical replicates.

#A5 plants with mutated *NPR1* (*npr1-1*, point mutation leading to destabilization of the protein) had similar growth defects as the #A5 parent (Figure 2.24). Unlike line #A5/*sid2-1*, no obvious difference in the growth development of both lines was observed, suggesting a lower influence of SA signaling downstream of *ICS1* on #A5. SA accumulation was not affected in #A5/*npr1-1* plants (Figure 2.25) consistent with results of previous studies which demonstrated wt-like SA level in *npr1* upon pathogen challenge (Delaney et al., 1995). Determining EDS1 levels by immunoblot analysis revealed reduced accumulation in #A5/*npr1-1* compared to #A5 (Figure 2.26). As in the case of *sid2-1*, also wt EDS1 level in *npr1-1* single mutant was lower than in Col-0 (preliminary data, EDS1 level in *npr1-1* analyzed once), suggesting a general effect of *npr1-1* on EDS1 accumulation.

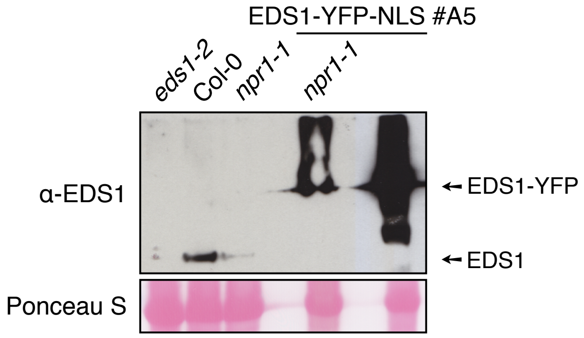

Figure 2.26: Reduced EDS1-YFP-NLS protein accumulation in plants lacking *NPR1*. Immunoblot analysis was performed on total protein extracts of 5-week-old plants. The membrane was probed with α-EDS1 antibody. Ponceau S staining of the membrane indicates equal loading and transfer onto the membrane. wt EDS1 protein and EDS1-YFP are labeled on the right.

To sum up, SA-mediated defense responses are an important and well-characterized element of EDS1 signaling. However, there are SA-independent branches of EDS1-mediated resistance which were also identified in this study. #A5 plants lacking crucial components of the SA signaling pathway still displayed dwarfism. Dwarfism is correlated with activated defense outputs as shown above, indicating SA-independent EDS1-triggered defense responses in these plants. Therefore, it can be concluded that the overall EDS1-YFP-NLS #A5 defense outputs are largely independent of the SA pathway.

2.7 Impact of PAD4 and SAG101 on EDS1-YFP-NLS phenotypes

EDS1 interacts with PAD4 and SAG101 (Feys et al., 2001, 2005; Rietz et al., 2011). PAD4 and SAG101 were described to stabilize EDS1 protein accumulation and there are indications for a signaling function of PAD4 and SAG101 in the EDS1 defense pathway (Feys et al., 2001, 2005). Therefore, I was interested in analyzing their role for the observed #A5 phenotypes. This will give further insights into the different functions of cytosolic and nuclear complexes of EDS1/PAD4 and EDS1/SAG101.

Thus, crosses were made to generate stable transgenic lines expressing EDS1-YFP-NLS #A5 protein and mutated versions of either *PAD4* (*pad4-1*) and *SAG101* (*sag101-1*) or both in the Col-0 *eds1-2* background, afterwards referred to as #A5/*pad4*, #A5/*sag101* and #A5/*pad4*/*sag101* (done by Jaqueline Bautor). #A5 plants lacking *PAD4* but with either functional or non-functional *SAG101* displayed a wt-like morphology (Figure 2.27A). Notably, plants with functional *PAD4* and *sag101-1* maintained growth defects similar to the #A5 parental line. Differences were quantified by measuring fresh weight and rosette sizes (Figure 2.27B). #A5 and #A5/*sag101* plants showed significantly reduced weight and decreased rosette diameter if grown at 22°C. Growth inhibition of #A5/*sag101* was suppressed in plants grown at 28°C, thus displaying the same temperature dependency of the developmental phenotype as #A5 plants. Alterations of the developmental phenotype

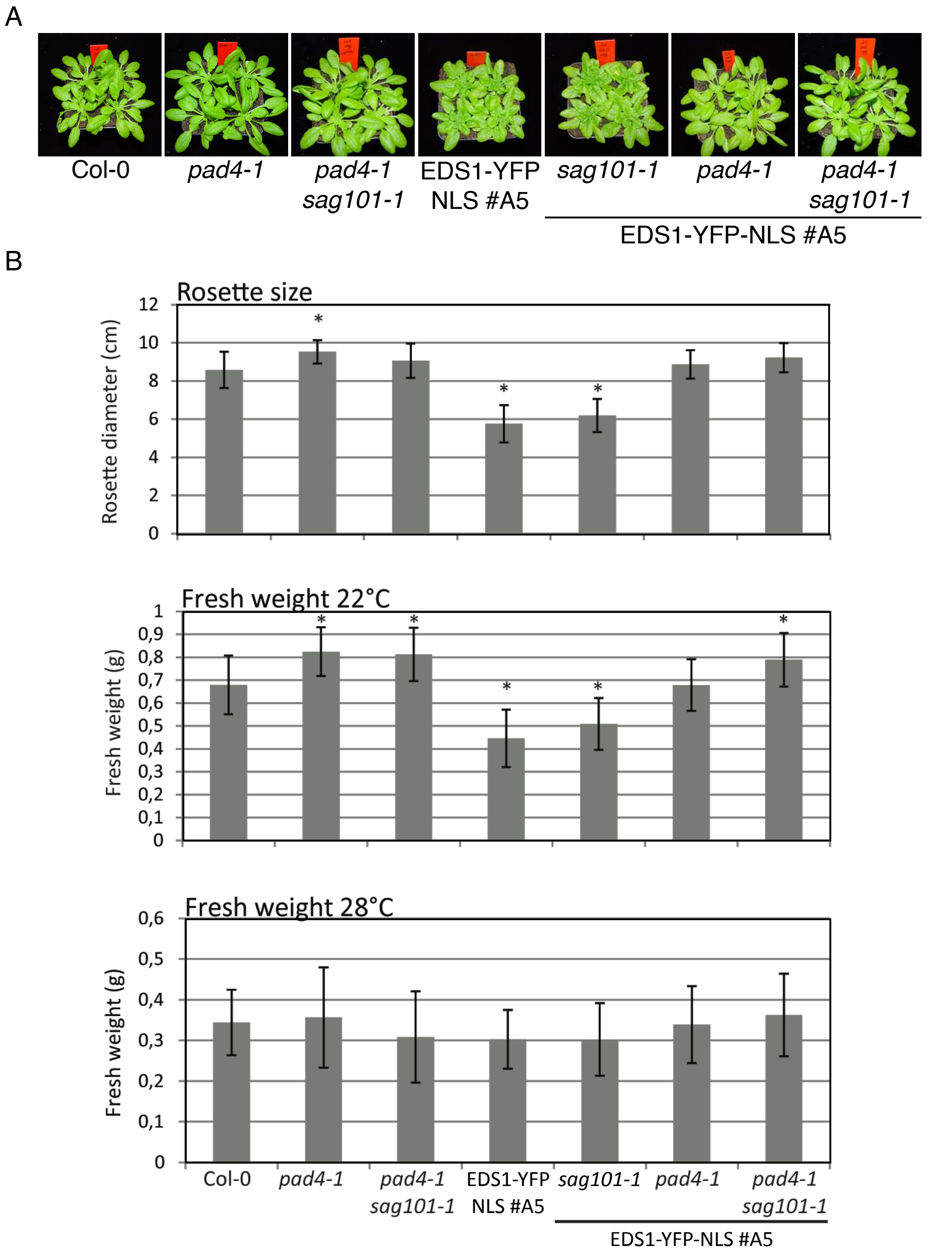

Figure 2.27: Loss of *PAD4* suppresses growth defects in EDS1-YFP-NLS #A5 plants (A) Growth phenotypes of EDS1-YFP-NLS #A5 lines in the *pad4-1* and *sag101-1* single or double mutant background. Pictures were taken of 5-week-old plants. **(B)** Quantification of growth defects of the depicted genotypes. Rosette size was measured of 5-week-old plants grown at 22°C. Error bars represent standard deviation of 16-20 single plants. Fresh weight was measured measured of 5-week-old plants grown at 22°C or 28°C. Error bars represent standard deviation of 20 single plants. Asterisks indicate significant differences compared to Col-0 (Student´s t-test, p-value < 0.01).

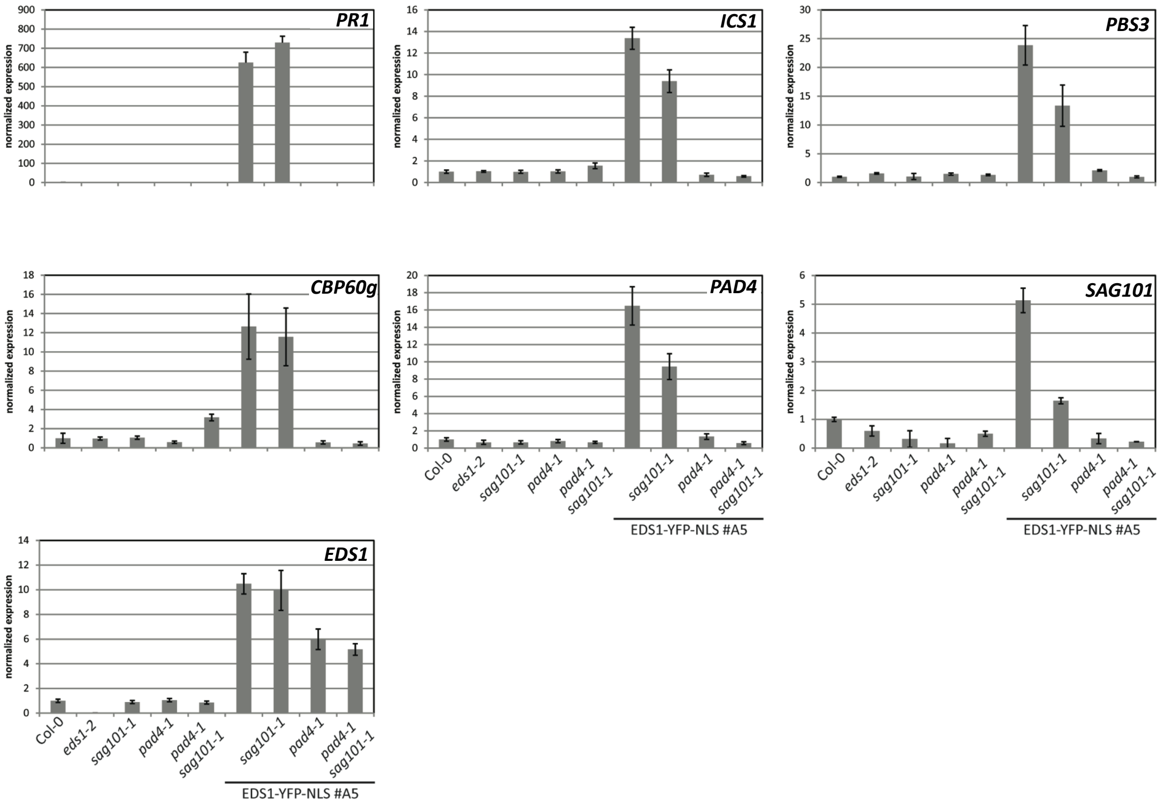

Figure 2.28: Transcriptional reprogramming in EDS1-YFP-NLS #A5 plants is suppressed by the loss of *PAD4*. Transcript levels were determined in leaf samples of 5-week-old plants and normalized using the internal control *UBIQUITIN*. Error bars represent standard deviation of 3 technical replicates. Similar results were obtained in three independent experiments.

only in lines with functional *PAD4* suggest that growth defects caused by high nuclear-restricted EDS1 accumulation are *PAD4*-dependent. *Sag101-1* did not reduce growth defects, indicating that the #A5-induced phenotypes are independent of *SAG101*.

As demonstrated above, growth defects in #A5 correlate with induction of defense outputs (see section 2.2). This prompted me to investigate transcriptional regulation of these lines. A set of common defense marker genes (*PR1*, *ICS1*, *PBS3*, *CBP60g*) was analyzed (Figure 2.28). Regulation of the selected genes in #A5/*sag101-1* and #A5 was similar. Thus, in #A5/*sag101* plants growth defects were accompanied by defense gene induction. Additionally, I examined *EDS1*, *PAD4* and *SAG101* transcript levels to study the impact of *PAD4* and/or *SAG101* on each other in the #A5 background. Elevated *EDS1* levels as monitored in #A5 were decreased in #A5/*pad4* and #A5/*pad4*/*sag101* but not in #A5/*sag101*, correlating with the loss of growth defects. In contrast, *pad4-1* and *sag101-1* single and double mutants in Col-0 wt background did not affect *EDS1* accumulation. Comparable transcript levels of *PAD4* were measured in #A5/*sag101* and #A5. There is a similar trend, but to a lower extent, for *SAG101* expression. From these analyses we can conclude that #A5 and #A5/*sag101* not only share developmental perturbation but also activated defense outputs.

Previous studies showed compromised resistance in *pad4-1* and hypersusceptibility of

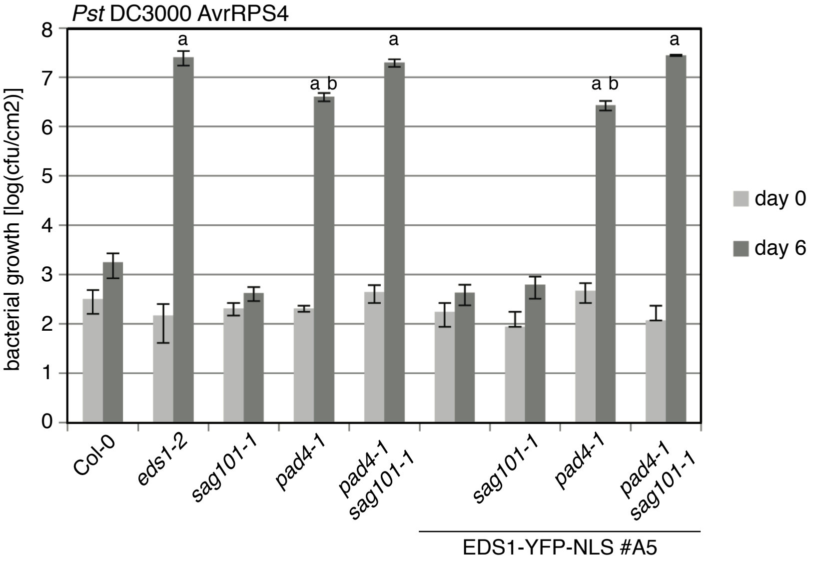

Figure 2.29: Loss of *PAD4* and *SAG101* contributes to enhanced susceptibility in the EDS1-YFP-NLS #A5 background upon avirulent pathogen challenge to the same extent as in wt background. Five-week-old plants of the indicated genotypes were spray-infected with *Pst* DC3000 AvrRps4. Bacterial titers were measured at 6 dpi. Error bars represent standard error of 4 technical replicates. Character a indicates significant difference to Col-0 at day 6, b indicates significant difference to *eds1-2* at day 6, calculated by Student´s t-test, p-value < 0.05. Similar results were obtained in three independent experiments.

pad4-1/*sag101-1* to a similar extent as in *eds1-2* (Feys et al., 2005; Lipka et al., 2005). This prompted me to test if excessive nuclear EDS1 activity affects defense in plants lacking *SAG101*, *PAD4* or both. Pathogen assays were performed to analyze bacterial growth in response to the avirulent bacterial pathogen *Pst* DC3000 AvrRps4. As shown above, #A5 conferred wt-like resistance and the same applied for plants expressing EDS1-YFP-NLS but lacking *SAG101* (Figure 2.29). Feys et al. (2005) already illustrated that *sag101-1* single mutants have no effect on plant immunity in wt background. Nevertheless, the strong susceptibility of *pad4-1*/*sag101-1* double mutants led them assume an intrinsic function of *SAG101* in defense signaling. #A5/*pad4* lines showed increased susceptibility to a similar extent as *pad4-1* single mutants. In addition, line #A5/*pad4*/*sag101* was as susceptible as *pad4-1*/*sag101-1* double mutants. Altogether, the experiment suggests that there is no specific influence of the excessive EDS1 activities on resistance of #A5/*pad4* and #A5/*sag101* since #A5 lines lacking *PAD4* or *SAG101* showed the same resistance response as their respective single or double mutants in wt background.

Feys et al. (2005) provided evidence that *PAD4* and *SAG101* assist EDS1 protein accumulation. Their data showed a 40 % decrease of EDS1 levels in *sag101-1* mutant plants and severely diminished EDS1 amounts in *pad4-1* (25 % of the levels in wt tissue). In *pad4-1*/*sag101-1* double mutants, EDS1 was hardly detectable. I analyzed the influence of *pad4-1* and *sag101-1* on EDS1 over-accumulation in #A5. A similar pattern of EDS1 accumulation as described in Feys et al. (2005) was detected, but with an increased starting level (Figure 2.30). Moreover, EDS1 protein amounts in the different lines corresponded to the measured *EDS1* transcript levels (Figure 2.28).

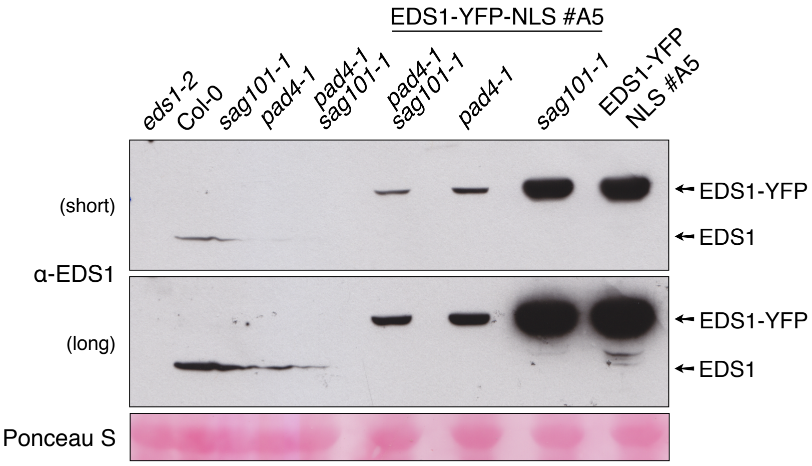

Figure 2.30: Altered EDS1 protein accumulation in *pad4-1* and *sag101-1* single or double mutants in the EDS1-YFP-NLS #A5 background. Immunoblot analysis showing expression of wt EDS1 and EDS1-YFP-NLS in the indicated genotypes. Total protein extracts were prepared from 5-week-old plants grown at 22°C and shifted for 1 week at 19°C to trigger EDS1-YFP-NLS #A5-induced defense outputs. Signal for some samples was only visible by long exposure (on the left), leading to overexposure of other samples (on the right). Ponceau S staining of the membrane indicates equal loading and transfer onto the membrane. wt EDS1 protein and EDS1-YFP are labeled on the right. Similar results were obtained in three independent experiments.

Combining these observations with the resistance phenotypes of the analyzed lines, a correlation between EDS1-YFP-NLS levels and resistance was observed; the lower EDS1 accumulation the less bacterial growth containment. Given the fact that line #B2 accumulates even lower amounts of EDS1 than #A5/*pad4* and #A5/*pad4*/*sag101* and still displayed wt-like resistance (Figures 2.1 and 2.19), increased susceptibility in these lines cannot be due insufficient levels of EDS1. Taking together, morphological and defense gene expression analyses indicate a requirement of *PAD4* for the #A5 phenotypes whereas *SAG101* has no influence.

2.8 Impact of wild type EDS1 protein on EDS1-YFP-NLS phenotypes

Results presented in this study as well as previous work (Garcia 2010) highlight the importance of balanced cytosolic and nuclear pools of EDS1 for plant growth development and resistance. Depletion of the nuclear EDS1 pool resulted in compromised basal and *R* gene-mediated resistance (García et al., 2010). Removal of cytosolic EDS1 *per se* did not cause any impairment in resistance, but massive amounts of nuclear EDS1 in the absence of cytosolic EDS1 leaded to growth defects, as shown in this study. This prompted me to test the impact of a relatively small pool of cytosolic EDS1 on the strong nuclear EDS1 over-accumulating line EDS1-YFP-NLS #A5. Therefore, nucleo-cytoplasmic wt EDS1 was crossed into #A5.

Interestingly, plants carrying wt EDS1 additionally to EDS1-YFP-NLS displayed wt-like development (Figure 2.31). I tested if nuclear localization of EDS1-YFP-NLS is influenced

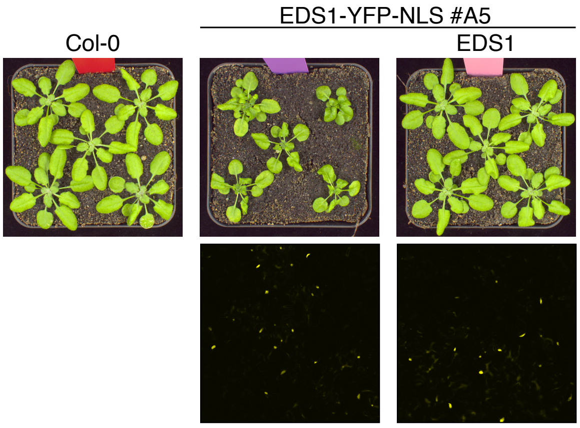

Figure 2.31: Introduction of wt EDS1 suppresses growth defects in EDS1-YFP-NLS #A5 plants but has no impact on EDS1-YFP-NLS subcellular distribution. Four-week-old plants of the depicted genotypes grown at 22° C were shifted for one week to 19° C to amplify EDS1-YFP-NLS #A5-induced defense. Confocal images of YFP fluorescence and bright field images of leaf epidermal tissue were taken of 3-week-old plants.

by wt EDS1, which is able to shuttle between both compartments (García et al., 2010). #A5 protein remained in the nucleus as revealed by CLSM (Figure 2.31). Wt EDS1 and EDS1-YFP-NLS levels were reduced in #A5/wtEDS1 lines compared to Col-0 and #A5, respectively (Figure 2.32). Both genes were driven by the native *EDS1* promoter. Therefore, a putative downregulation at the transcriptional level should encounter EDS1 and EDS1-YFP-NLS #A5 accumulation and will not influence overall imbalance. Furthermore, a silencing effect on the transgene can be excluded because similar transcript levels were monitored in #A5 and #A5/wtEDS1 (Figure 2.33). A possible explanation for the suppression of growth defects in #A5/wtEDS1 is that cytosolic EDS1 counteracts excessive nuclear EDS1 activity by decreasing EDS1 protein level in general and therefore, the threshold to induce growth defects may not be reached anymore. Alternatively, even though there is still strong imbalance between both pools of EDS1, specific function of cytosolic EDS1 is able to antagonize nuclear activity. Nucleo-cytoplasmic shuttling of EDS1 and thereby triggered processes might be sufficient to abolish excessive nuclear activity. *EDS1* transcript levels were similar in both lines, indicating a reduction of EDS1 in #A5/wtEDS1 by post-transcriptional mechanisms. It should be noted that measured transcript levels represent wt *EDS1* and the transgene (Figure 2.33). Inhibition of growth defects is accompanied by the loss of defense gene induction of *PR1* and *PBS3* (Figure 2.33), suggesting that wt EDS1 influences not only plant development but also affects defense outputs.

Taking together, cytosolic EDS1 activity or the ability of nucleo-cytoplasmic shuttling is able to suppress #A5 phenotypes. However, reduced levels of EDS1-YFP-NLS might be another potential explanation for the loss of defense outputs. Comparison of EDS1-YFP-NLS protein amounts in #A5/wtEDS1 and #A5/*sid2-1* revealed similar accumulation in both lines. #A5/*sid2-1* displayed growth defects, suggesting that suppression of defense outputs in #A5/wtEDS1 is unlikely to due to insufficient amounts of EDS1-YFP-NLS protein.

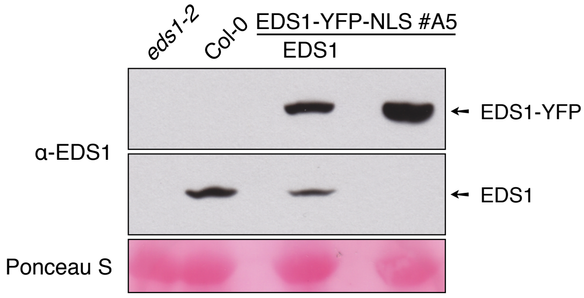

Figure 2.32: Reduced EDS1 protein accumulation in EDS1-YFP-NLS #A5 plants expressing wt EDS1. Immunoblot analysis was performed on total protein extracts of 5-week-old plants grown at 22°C and shifted for one week to 19°C to trigger EDS1-YFP-NLS #A5-induced defense outputs. The membrane was probed with α-EDS1 antibody. Ponceau S staining of the membrane indicates equal loading and transfer onto the membrane. Migration of wt EDS1 and EDS1-YFP is marked by arrows on the right. Similar results were obtained in two independent experiments.

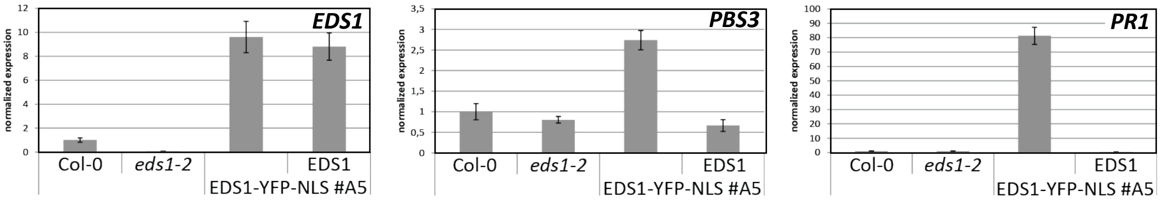

Figure 2.33: Introduction of wt EDS1 inhibits defense marker genes expression in EDS1-YFP-NLS #A5 plants. Transcript levels were determined in leaf samples of 5-week-old plants grown at 22°C after shifting for one week to 19°C to amplify EDS1-YFP-NLS #A5-induced defense outputs. Transcript expression was normalized using the internal control *UBIQUITIN*. Error bars represent standard deviation of 3 technical replicates.

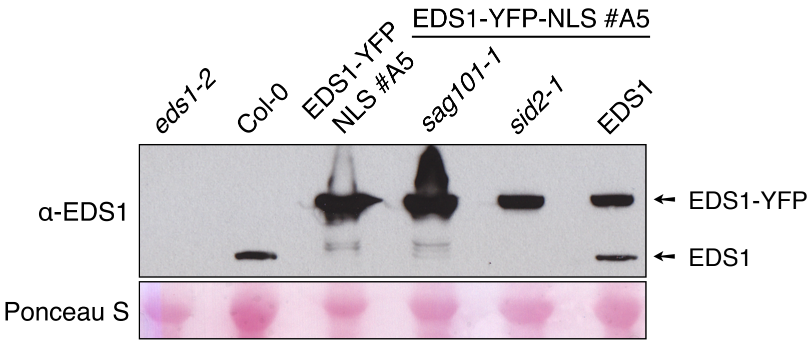

Figure 2.34: EDS1-YFP-NLS protein accumulation in plants lacking *SAG101* or *ICS1* or expressing wt EDS1. Immunoblot analysis was performed on leaf tissue of 5-week-old plants grown at 22°C and shifted for 1 week at 19°C to trigger EDS1-YFP-NLS #A5-induced defense. Total protein extracts were prepared from leaf tissue. The membrane was probed with α-EDS1 antibody. Ponceau S staining was performed to ensure equal loading. Migration of wt EDS1 and EDS1-YFP is marked by arrows on the right. Similar results were obtained in two independent experiments.

2.9 Conditional accumulation of EDS1 in the nucleus

By investigating EDS1-YFP-NLS lines, the effect of prolonged nuclear EDS1 accumulation was studied. As observed for line #A5 and #A3, this can cause secondary effects such as reprogramming of genes not directly linked to EDS1 activity potentially due to artificial high nuclear amounts of EDS1 and/or YFP or morphological abnormalities (Figure 2.3). Therefore, I investigated immediate effects of nuclear EDS1 accumulation and compared it to the defense outputs in plants with permanent nuclear-restricted EDS1 levels. For this purpose, two systems for conditional induction were tested.

2.9.1 Estradiol-induced nuclear EDS1 accumulation

Stable transgenic lines in the Col/*eds1-2* background were generated that expressed EDS1-YFP-NLS/nls driven by an estradiol-inducible promoter, hereafter referred to as pED:EDS1-YFP-NLS/nls (Zuo et al., 2001). Two homozygous pED:EDS1-YFP-NLS lines (#13 and #25) with similar levels of EDS1-YFP-NLS protein 24h after estradiol treatment compared to EDS1 levels in Col-0 were selected for further analysis (Figures 2.36 and 2.37). Lines pED:EDS1-YFP-nls #8 and #17 were chosen as negative control. They displayed higher amounts of EDS1-YFP-NLS protein compared to EDS1 in Col-0 but similar levels as line EDS1-YFP-NLS #A5 (Figure 2.36).

Next, resistance of the estradiol-inducible lines in response to avirulent *Pst* DC3000 AvrRps4 was studied. Bacteria were surface-inoculated 24h after spraying with estradiol (10μM) or DMSO (mock) and bacterial proliferation was measured 3 dpi (Figure 2.35). Both pED:EDS1-YFP-NLS lines showed hypersusceptibility with or without estradiol treatment to a similar extent as *eds1-2*. pED:EDS1-YFP-nls #8 plants exhibited intermediate resistance compared to resistance in Col-0 and EDS1-YFP-NLS/ #A5 plants and hypersusceptible *eds1-2*. Estradiol application itself did not affect pathogen growth illustrated by the fact that bacteria grew to the same extent with or without estradiol treatment on Col-0 and *eds1-2*. This observation suggests that nuclear EDS1 accumulation for only a short period of time is insufficient to fulfill its function in resistance. An alternative explanation would be a strong reduction of EDS1 levels over the time of infection. To test this scenario, I monitored EDS1-YFP-NLS/nls levels for 6 days upon estradiol treatment (Figure 2.36). EDS1 accumulated after 24h in all estradiol-inducible lines and remained detectable at 48h and 72h after estradiol application in lines NLS #25, nls #8 and nls #17. After 6 days, there is still protein detectable in pED:EDS1-YFP-nls lines #8 and #17. Only line pED:EDS1-YFP-NLS #13 showed no protein accumulation later than 24h after spray-induction. However, EDS1 protein in Col-0 was also not detectable at the 72h time point. Immunoblot results of protein expression for at least 6 days were confirmed by confocal microscopy analysis, showing YFP signal in estradiol-inducible plants for more than a week after treatment (data not shown). Nevertheless, to be sure that there is always an adequate amount of EDS1 in the cell and to answer the question if some more days of EDS1-YFP-NLS activity would be enough to restore wt resistance, I applied estradiol for three times in a higher dosage (20μM instead of 10μM used before) over a period of 5 days. The last estradiol treatment was performed shortly before *Pst* DC3000 AvrRps4 inoculation to assure sufficient protein levels during the entire time of infection. EDS1 was detected at all tested time points (Figure 2.37). However, pED:EDS1-YFP-NLS plants still displayed hypersusceptibility (data for pED:EDS1-YFP-nls is missing), suggesting that also 5 days of nuclear EDS1 protein accumulation is not sufficient to induce resistance (Figure 2.38).

Furthermore, localization of estradiol-inducible EDS1-YFP-NLS/nls protein was studied (Figure 2.39). YFP signal was exclusively detected in nuclei in line NLS #13. Line NLS #25 predominantly displayed nuclear EDS1 localization and nls #17 plants showed nucleo-cytoplasmic distribution. I recognized that EDS1-YFP-NLS/nls mostly accumulated along leaf veins (Figure 2.40). A possible explanation is that due to spray application of estradiol, it enters through stomata into the mesophyll and is directly transported into the vascular bundle. Thus, no enhanced resistance after estradiol application might be observed because the measured protein is not equally distributed in the whole leaf. Given this, EDS1 might not fulfill its function in all cells, overall resulting in hypersusceptible plants.

Estradiol-inducible plants expressing EDS1-YFP without any NLS/nls tag will be tested for their complementation in resistance to rule out effects due to the estradiol-inducible promoter. Generation of plants carrying this construct is in progress. If they show increased susceptibility, it would point to non-functional *EDS1* due to the estradiol-inducible promoter. If plants containing this transgene display wt-like resistance, it suggests that prolonged nuclear accumulation might be required for full resistance. Notably, pED:EDS1-YFP-nls lines displayed intermediate resistance, indicating some EDS1 activity.

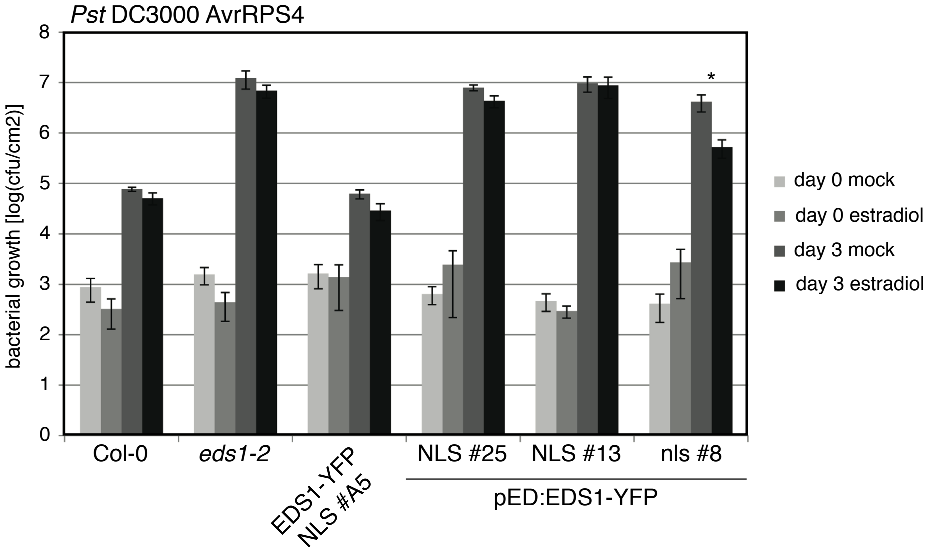

Figure 2.35: Plants upon estradiol-induced expression of EDS1-YFP-NLS protein are susceptible to avirulent pathogens. Four-week-old plants of the indicated genotypes were spray-infected with *Pst* DC3000 AvrRps4 24h after estradiol or mock treatment. Bacterial titers were measured at 3 dpi. Bacterial entry was determined at 4 hpi. Error bars represent standard error of 3 technical replicates. Asterisk indicates significant difference between mock and estradiol treatment of bacterial titers 3 dpi (Student´s t-test, p-value < 0.05). The experiment was repeated 3 times with similar results.

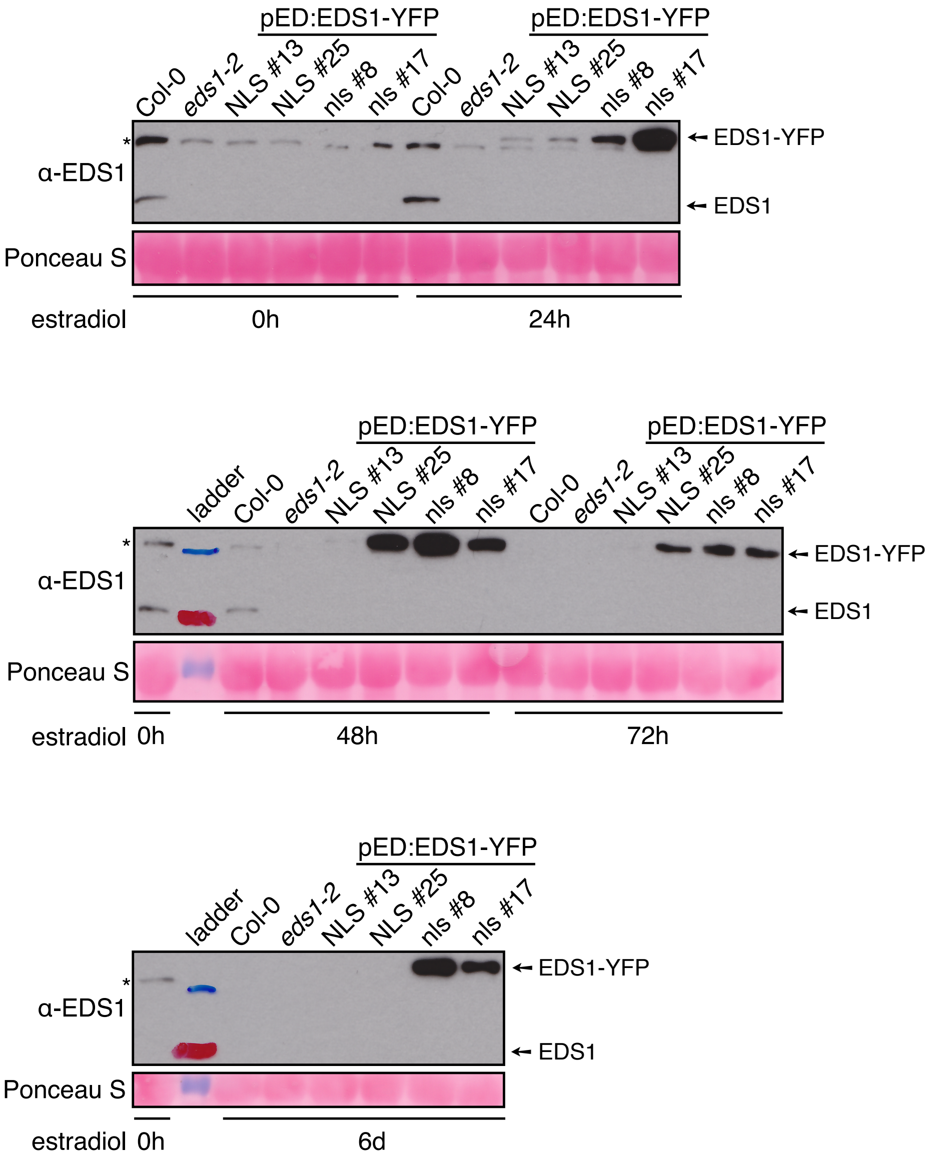

Figure 2.36: EDS1-YFP-NLS/nls protein accumulates upon estradiol application. Four-week-old plants of the depicted genotypes were spray-induced with estradiol. Leaf samples were taken at the indicated time points. Immunoblot analysis was performed of total protein extracts. Membrane was probed with α-EDS1 antibody. Ponceau S staining of the membrane indicates equal loading and transfer onto the membrane. Migration of wt EDS1 and EDS1-YFP is marked by arrows on the right. * : nonspecific cross-reacting signal.

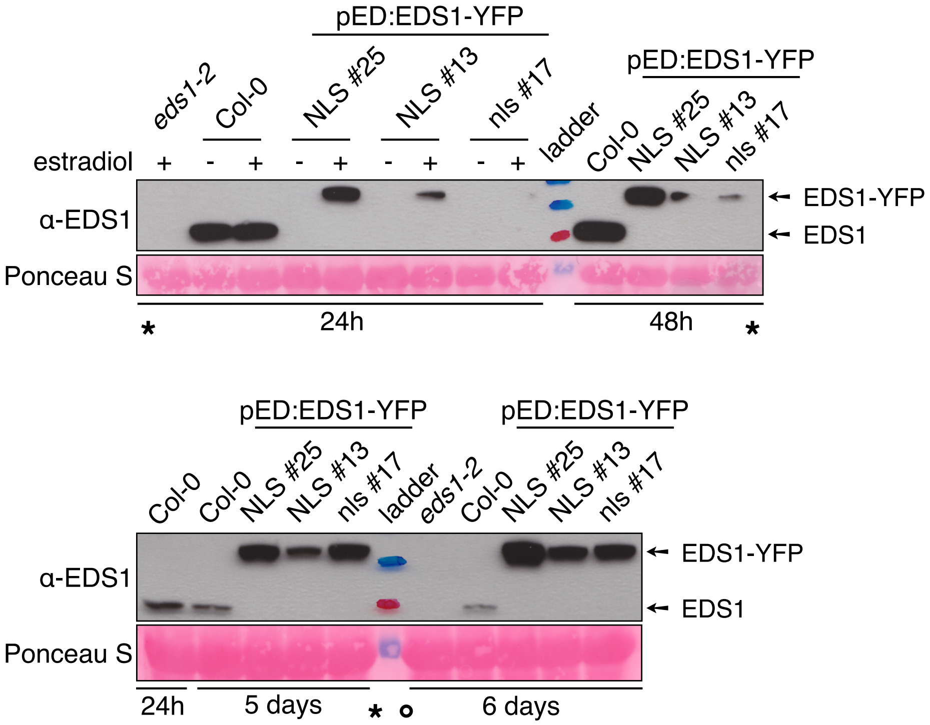

Figure 2.37: EDS1-YFP-NLS protein levels at different time points after estradiol application. Immunoblot analysis was performed on leaf extracts of 4-week-old plants after estradiol or mock treatment at the indicated time points. The membrane was probed with α-EDS1 antibody. Ponceau S staining of the membrane indicates equal loading and transfer onto the membrane. Application of estradiol is indicated by asterisks. Time point of spray-inoculation with *Pst* DC3000 AvrRps4 is represented by a circle. Migration of wt EDS1 and EDS1-YFP is marked by arrows on the right.

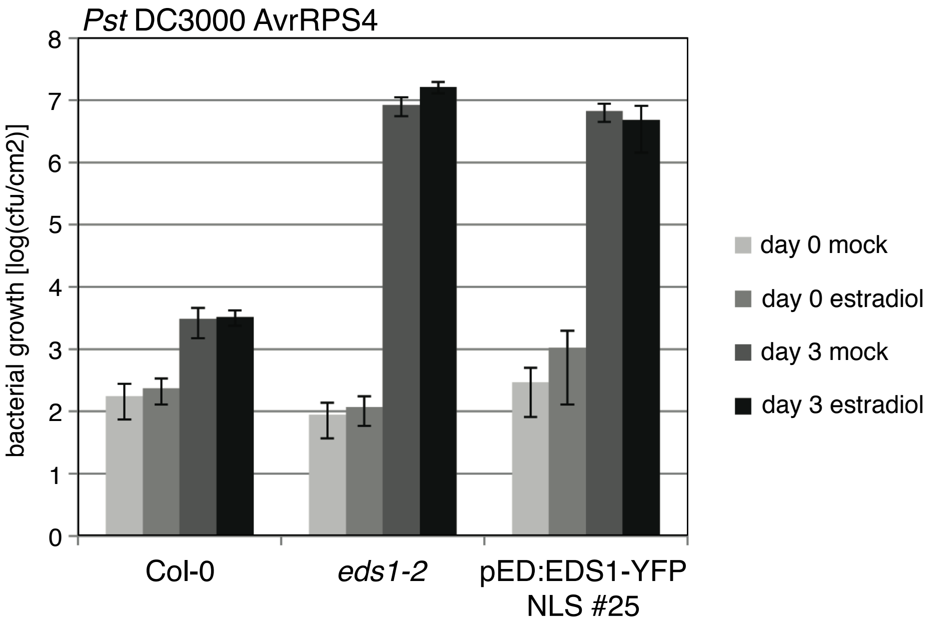

Figure 2.38: Plants after five days of constitutive EDS1-YFP-NLS protein expression are hypersusceptible to avirulent pathogens. Four-week-old plants were treated 3 times with estradiol before spray-infection with *Pst* DC3000 AvrRps4 (see Figure 2.37). Bacterial titers were measured at 3 dpi. Bacterial entry was determined at 4 hpi. Error bars represent standard error of 3 technical replicates.

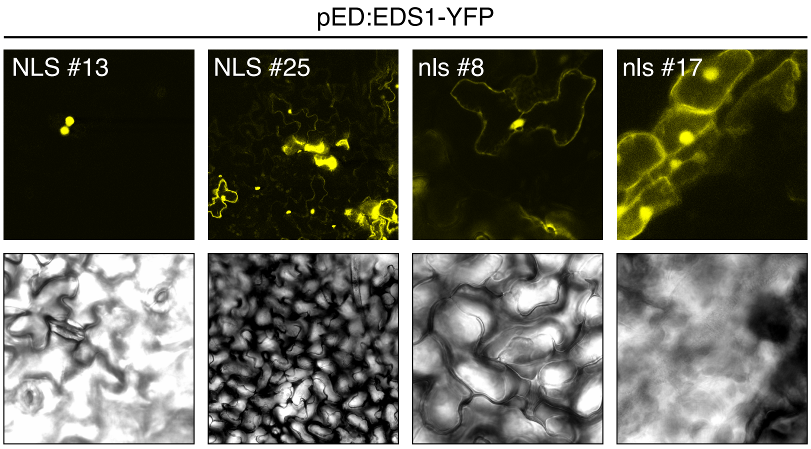

Figure 2.39: Subcellular distribution of estradiol-induces EDS1-YFP-NLS expression. Confocal images of YFP fluorescence in leaf epidermal tissue of pED:EDS1-YFP-NLS lines after constitutive estradiol spray-application. Confocal and bright field images were taken of 3-week-old soil-grown plants.

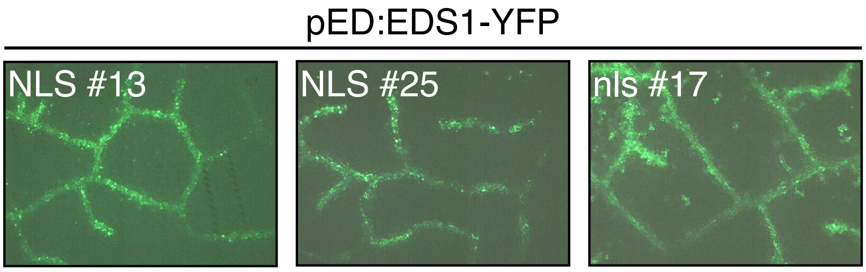

Figure 2.40: EDS1-YFP-NLS protein accumulates preferentially in leaf veins upon estradiol treatment. Confocal images of YFP fluorescence in pED:EDS1-YFP-NLS lines after three times of estradiol spray-application within 7 days. Pictures were taken of leaf epidermal tissue of 4-week-old soil-grown plants.

In summary, despite the presence of EDS1-YFP-NLS in estradiol-inducible lines, plants did not show resistance after short-term expression of nuclear EDS1. This is a surprising result given the fact that García et al. (2010) showed full defense responses shortly after allowing nuclear EDS1 accumulation in EDS1-GR lines upon Dex treatment. One possible explanation might be a requirement of prolonged EDS1 activity, either cytoplasmic or nuclear, to confer resistance. Alternatively, slow transcription or translation might be needed to allow proper folding and maturation of EDS1 or for the assembly of EDS1 complexes.

2.9.2 Temperature-induced nuclear EDS1 accumulation

In a second approach to study immediate effects of nuclear EDS1 activity, I made use of the temperature dependency for protein accumulation in EDS1-YFP-NLS lines driven by the native *EDS1* promoter. As described above, at 28°C, EDS1-YFP-NLS #A5 showed moderate nuclear EDS1 levels and the growth abnormalities were suppressed (Figures 2.12, 2.11). At 19°C, growth defects became amplified (Figure 2.14). Therefore, #A5 plants were grown at 28°C to avoid excessive nuclear EDS1 activities. After 3 weeks, plants were transferred to 19°C to induce EDS1-dependent responses.

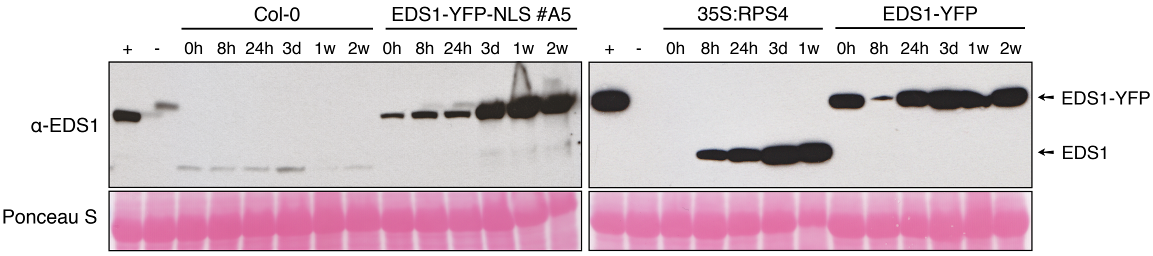

Figure 2.41: Induced EDS1 protein accumulation after temperature shift to 19°C. Plants of the depicted genotypes were grown for three weeks at 28°C and then shifted to 19°C. Leaf samples were harvested at the indicated time points after shift. Immunoblot analysis was performed on total protein extracts. Membranes were probed with α-EDS1 antibody. Ponceau S staining was performed to ensure equal loading. Migration of wt EDS1 and EDS1-YFP is marked by arrows on the right. Similar results were obtained in two independent experiments. Membrane comparison controls: + : EDS1-YFP plants 24h after shift; - : 35S:RPS4/*eds1-2* 24h after shift.

First, protein accumulation after temperature shift was analyzed. Besides the tested #A5, Col-0 and EDS1-YFP lines, plants were included that overexpress the TIR-NB-LRR protein RPS4 (35S:RPS4) and 35S:RPS4/*eds1-2* as positive and negative controls, respectively. It is a well-established system in our group to study RPS4 activated defense signaling. After shifting plants to low temperature, RPS4 signaling pathway is rapidly activated and induces defense-like EDS1-dependent transcriptional reprogramming (Blanvillain-Baufumé et al., unpublished). EDS1 protein in 35S:RPS4 was not detected before temperature shift but it increased early after shift and maintained high levels throughout the analyzed time points (Figure 2.41). One week after shift, 35S:RPS4 plants were strongly chlorotic and died before two-week samples could be taken. In contrast, #A5 exhibited protein accumulation already before plants were shifted. They also showed increasing levels after the shift. Strong elevated EDS1 amounts were measured starting from day 3 onwards. Similar to 35S:RPS4, #A5 plants produced yellowish leaves after 1 week, but to a lower extent, so that sampling of 2-week old plants was still possible. EDS1 levels in Col-0 increased slightly until 3 days after shift (Figure 2.41). Col-0 plants *per se* are responsive to temperature illustrated by reduced EDS1 expression when grown at high temperature (Figure 2.12). EDS1-YFP levels were not affected by temperature, in agreement with my previous observation (Figure 2.12). The reduced EDS1-YFP accumulation detectable 8h after switch is assumed to be an experimental mistake and was not observed in a repeated experiment. Additionally, protein localization was monitored after shift to test a potential effect of temperature on protein distribution. EDS1-YFP-NLS was found inside nuclei and EDS1-YFP was localized in cytosol and nucleus as expected (Figure 2.42), suggesting that temperature has no influence on EDS1 distribution. I analyzed transcript levels of selected genes before, 3h, 8h, 24h and 3 days after shifting plants to 19°C. *PR1* and *PBS3* transcript levels were elevated at 8h after shift in 35S:RPS4; *PR1* massively increased until 1 week after shift when plants were almost dead, whereas *PBS3* expression peaked at 8h and decreased afterwards (Figure 2.43). By contrast, #A5 plants showed induction of *PR1* and *PBS3* not until 3 days after shift and maintained elevated levels in week 1 and 2 after temperature shift. Thus, induced defense gene expression in #A5 and 35S:RPS4 correlates with increased

EDS1 accumulation. There was no detectable influence of temperature on the tested gene regulation in Col-0 and EDS1-YFP (Figure 2.43), which was also reflected by constant EDS1 protein levels in these lines (Figure 2.41).

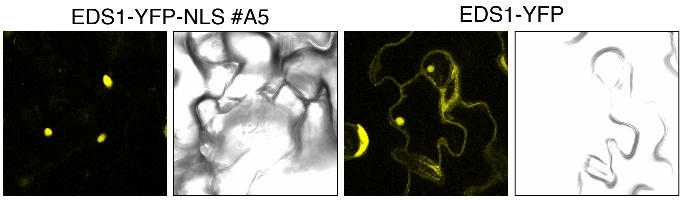

Figure 2.42: Temperature shift does not change EDS1-YFP subcellular distribution. Confocal images of YFP fluorescence in leaf epidermal tissue. Confocal and bright field images were taken 3 days after shifting plants from 28°C to 19°C.

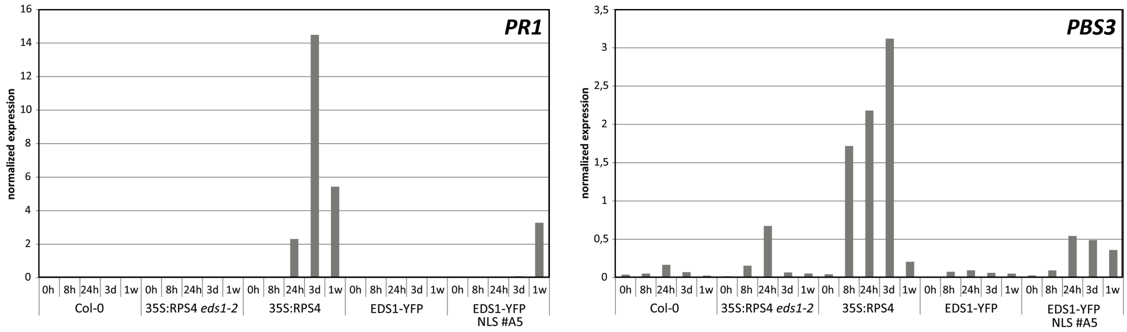

Figure 2.43: Low temperature induces transcriptional reprogramming in EDS1-YFP-NLS #A5 plants. Transcript levels of *PR1* and *PBS3* were determined in leaf samples at the indicated time points after shifting plants from 28°C to 19°C. Gene expression was normalized using the internal control *UBIQUITIN*. The experiment was repeated twice with similar results.

These results indicate a slower response of EDS1-YFP-NLS #A5 on temperature compared to the rapid 35S:RPS4 system, possibly due to a lag phase until sufficient nuclear EDS1 accumulation is reached to induce transcriptional changes. This might be due to the lack of TIR-NB-LRR activation in EDS1-YFP-NLS #A5. Thus, the temperature system cannot be used for studying immediate effects of high EDS1 nuclear activity as initially planned. Moreover, these observation support the threshold theory mentioned above, stating that a certain level of unbalanced nuclear-restricted EDS1 has to be passed before defense outputs start to occur (section 2.1).

In summary, the immediate resistance phenotype did not correspond to the observed effects of prolonged nuclear-restricted EDS1 accumulation. By investigating resistance responses, hypersusceptibility of estradiol-inducible lines is in sharp contrast to wt-like resistance in EDS-YFP-NLS lines driven by the native *EDS1* promoter. This suggests that short-term induction of nuclear EDS1 is insufficient to confer resistance.

2.10 Identification of nuclear EDS1 protein associations

Recent studies revealed new EDS1 associations in addition to the known interactions with its signaling partners PAD4 and SAG101. EDS1 was found in complexes with TIR-NB-LRR proteins and the negative immune regulator SRFR1 (Heidrich et al., 2011; Bhattacharjee et al., 2011; Kim et al., 2012). Furthermore, preliminary data suggests an association of EDS1 with components of the transcriptional machinery such as transcription factors and chromatin remodeling proteins (T. Griebel, S. Blanvillain-Baufumé, M. Muhr, R. P. Huibers; unpublished data). RPS4 is one of the TIR-NB-LRR proteins identified to reside in complex with EDS1 (Heidrich et al., 2011; Bhattacharjee et al., 2011). New data revealed chromatin association of RPS4. There are indications that RPS4 is involved in transcriptional dynamics of defense genes. Chromatin-immunoprecipitation of RPS4 coupled with DNA sequencing to identify DNA binding sites of RPS4 suggest that 40 % of the genes targeted by RPS4 might be regulated in an EDS1-dependent manner, further suggesting a tight connection of EDS1 with transcriptional regulation (Blanvillain-Baufumé et al., unpublished). As reported in García et al. (2010), nuclear EDS1 is necessary for transcriptional reprograming of defense genes, emphasizing its role in the nucleus. Therefore, identification of new nuclear EDS1 interaction partners will help to gain further insights into the precise EDS1 mode of action in plant immunity.

EDS1-YFP-NLS #A5 plants were used as a tool to discover potential new nuclear associations of EDS1 since they exhibit several advantages for this approach. First, they express high levels of EDS1 only present in the nucleus, the compartment where transcription takes place. Second, 5-week-old #A5 plants showed induced defense outputs without requirement of a pathogen trigger (Figures 2.7, 2.8). Defense responses are supposed to be activated uniformly in all plant cells, suggesting most EDS1 bound in complexes necessary for its activity during defense. For the analysis, plant leaf material of 5-week-old plants was used. At that age, plants started to show growth defects which correlate with activation of defense responses. Induction of defense outputs were confirmed by examining expression of defense marker genes. Plants expressing high levels of YFP alone were used as negative control. Due to the small size of YFP, it can freely move within the cell. Massive amounts of YFP could be detected in cytosol and nucleus. This control was used to discard false positive hits which bind specific to YFP. EDS1-YFP-NLS protein was pulled-down using α-GFP beads. To identify putative interaction partners, gel-free mass spectrometry was chosen. Associations of EDS1, e.g. with components of the transcriptional machinery, might be rare and transient and therefore possibly not detectable by commonly used two-dimensional gel separation methods. Indeed, no striking differences in the composition of proteins eluted after immunoprecipitation was detected by coomassie staining in #A5 compared to the negative control (Figure 2.45). PAD4, as a known EDS1 interaction partner was detected in the eluate after co-immunoprecipitation, demonstrating that the applied method is effective (Figure 2.44).

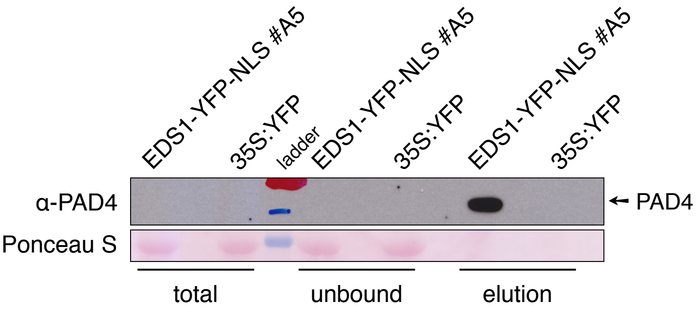

Figure 2.44: Co-Immunoprecipitation of PAD4 with EDS1-YFP-NLS #A5. Co-IP with α-GFP beads from total protein extracts of EDS1-YFP-NLS #A5 and 35S:YFP plants. Crude extract (total), flow-through (unbound) and immunoprecipitated (elution) protein fractions were subjected to SDS-PAGE. Immunoblot analysis with α-PAD4 antibody. Ponceau S staining was performed to ensure equal loading.

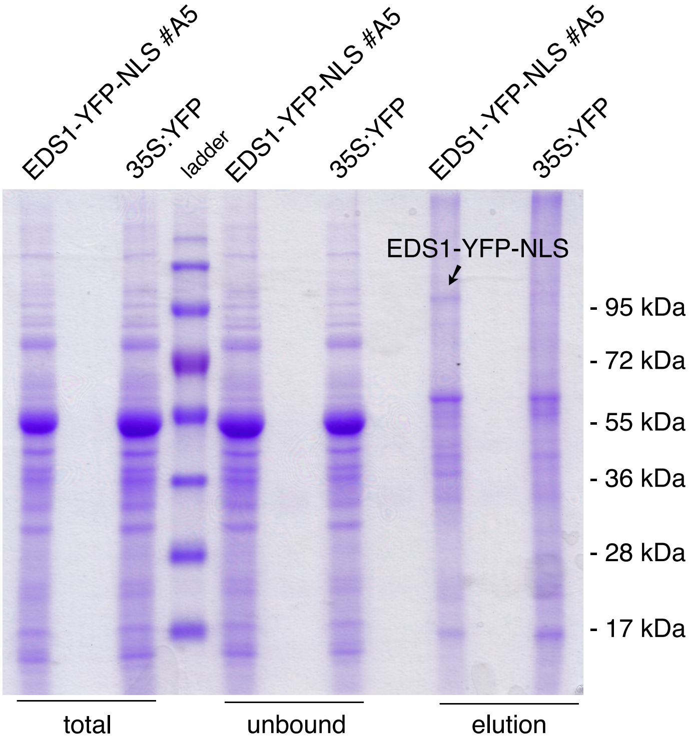

Figure 2.45: Co-Immunoprecipitation of putative nuclear EDS1 interaction partners. Co-IP with α-GFP beads from total protein extracts of EDS1-YFP-NLS #A5 and 35S:YFP plants. Crude extract (total), flow-through (unbound) and immunoprecipitated (elution) protein fractions were subjected to SDS-PAGE and stained with Coomassie Blue.

Stringent data analysis after LC-MS/MS combined with Orbitrap mass analysis revealed a list of 12 putative nuclear EDS1 interactors (Table 2.3). The by far most significant proteins identified are the known EDS1 signaling partners PAD4 and SAG101. One potentially interesting EDS1 interactor candidate is RPN2, which is part of the 26S proteasome. Recent studies indicate a role of the 26S proteasome in plant defense as shown for RPN1a, RPT2a and RPN8a, three further subunits of the 26S proteasome (Chung and Tasaka, 2011; Yao et al., 2012). Additionally, RPN2 was discovered in an independent mass spectrometry analysis using different transgenic lines as starting material to identify putative cytosolic and

Table 2.3: List of putative EDS1 associations identified by LC-MS/MS combined with Orbitrap mass analysis. Identification of candidates interacting with nuclear EDS1 using the ProteinScape bioinformatic platform. Analysis was performed of 3 biological replicates. Replicate 1 and 2 were run on a 15 cm column whereas replicate 3 was run on a 10 cm column due to technical circumstances. Predicted subcellular localization according to TAIR database.

AT gene ID	Gene description	predicted nuclear localization	Average spectral count	identified in replicates #
AT3G52430	PHYTOALEXIN DEFICIENT 4 (PAD4)	yes	33	1, 2, 3
AT5G14930	SENESCENCE-ASSOCIATED GENE 101 (SAG101)	yes	21	1, 2, 3
AT2G32730	19S regulatory subunit of 26S proteasome (RPN2)	yes	9	1, 2, 3
AT2G33210	HEAT SHOCK PROTEIN 60-2 (HSP60-2)	no	7	1, 2, 3
AT2G41840	Ribosomal protein S5 family protein	yes	8	1, 2, 3
AT5G42080	DYNAMIN-LIKE PROTEIN (DL1)	no	7	1, 2
AT1G09780	Phosphoglycerate mutase 1	no	6	1, 2
AT3G25530	Gamma-hydroxybutyrate dehydrogenase (GHBDH)	yes	6	1, 2
AT3G04120	GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C SUBUNIT (GAPC1)	yes	3	1, 2, 3
AT4G39200	Ribosomal protein S25 family protein	yes	5	1, 2, 3
AT2G27030	CALMODULIN 5 (CAM5)	yes	5	1, 2
AT3G13920	EUKARYOTIC TRANSLATION INITIATION FACTOR 4A1 (EIF4A1)	yes	2	1, 2

nuclear EDS1/PAD4 interactors (H. Cui, personal communication), supporting its association with EDS1. Several proteins identified are involved in general stress responses, such as HSP60-2, and more specific in abiotic stress responses (phosphoglycerate mutase 1, GHBDH, GAPC1, CAM5 (Amme et al., 2006; Allan et al., 2009; Al-Quraan et al., 2010; Guo et al., 2012)). It has to be noted that some of the interacting proteins are not predicted to be nuclear. The recently described EDS1 associations with RPS4, RPS6, SRFR1 and VICTR (Heidrich et al., 2011; Bhattacharjee et al., 2011; Kim et al., 2012) were not found under the used conditions. Moreover, only one of the detected proteins, EIF4A1, is connected to the transcriptional machinery (Loh et al., 2009; Chang et al., 2009).

In summary, ten new candidate proteins potentially interacting with EDS1 were identified. RPN2 is one promising putative interactor given the fact that it was also found in a parallel independent study. In addition, RPN2 is part of a complex which was previously reported to play a role in plant defense (Yao et al., 2012). The further identified putative EDS1 interactors might help to elucidate the mechanisms underlying EDS1 activity in immunity.

3 Discussion

Studies published during the last years emphasize the nucleus as the key compartment for successful activation of defense signaling in plant immunity. Several R proteins were demonstrated to require nuclear localization to launch defense responses (Shen et al., 2007; Wirthmueller et al., 2007; Burch-Smith et al., 2007; Cheng et al., 2009). Furthermore, entering of the pathogen effector AvrRps4 into the nucleus was shown to be necessary for defense activation and resistance (Heidrich et al., 2011). Additionally, NPR1, a defense signaling components important for SA-mediated activation of SAR, accumulates in the nucleus upon pathogen-induced cellular redox changes to fulfill its function in regulating defense gene expression (Zhang et al., 1999; Mou et al., 2003). Transcriptional reprogramming was demonstrated to be crucial for defense activation and resistance (Moore et al., 2011).

At the same time, a growing body of evidence points to an important function of EDS1 in the nucleus. Exclusion of nuclear EDS1 inhibited transcriptional reprogramming upon pathogen challenge and plants were impaired in resistance (García et al., 2010). Studies of Heidrich et al. (2011) and Bhattacharjee et al. (2011) revealed an interaction of nuclear EDS1 with the TIR-NB-LRR protein RPS4. Recent data point to an association of RPS4 with chromatin of defense-related genes in an EDS1-dependent manner (Blanvillain-Baufumé et al., unpublished). Based on these findings, it is reasonable to hypothesize that nuclear activity of EDS1 is essential in EDS1-mediated plant immunity.

Despite the progress achieved over the past years, the mode of action of EDS1 still remains elusive. EDS1 is known to act downstream of TIR-NB-LRR protein activation and upstream of defense responses such as defense gene induction, SA accumulation or cell death initiation (Zhang et al., 2003; Wirthmueller et al., 2007). The focus of this study was to gain further insight into how EDS1 coordinates multiple defense outputs and how this process is regulated by the localization of EDS1 to distinct cellular compartments.

3.1 Nuclear EDS1 is sufficient to confer resistance

EDS1 is a nucleo-cytoplasmic protein (Feys et al., 2005). Previous studies reported an increase of the nuclear EDS1 pool early upon pathogen inoculation which became equilibrated at later time points (García et al., 2010). Early nuclear enrichment during infection supports the functional significance EDS1 in the nucleus to activate defense responses. To shed light on how EDS1 localization affects plant resistance and to analyze the function of nuclear EDS1, stable transgenic lines with enforced nuclear EDS1 by fusion to the nuclear localization sequence of the SV40 large T antigen (EDS1-YFP-NLS lines) were characterized.

Strikingly, EDS1-YFP-NLS plants which accumulate nuclear EDS1 levels similar to Col-0 (Figure 2.4) behaved wt-like in all tested defense outputs (Figures 2.7, 2.8, 2.10B). Furthermore, their resistance to virulent (*H. arabidopsidis* Noco2, *Pst* DC3000) and avirulent (*Pst* DC3000 AvrRps4) pathogens did not differ from wt plants (Figure 2.19), suggesting sufficiency of nuclear EDS1 activity to restrict pathogen growth.

At the first glance it is a surprising result since previous data indicated a requirement of both - the cytoplasmic and nuclear pool - for complete resistance (García et al., 2010). This conclusion was based on experiments with transgenic lines in which EDS1 is restricted to the cytoplasm either by increasing its nuclear export by attaching a nuclear export signal (NES) or via fusion to a glucocorticoid receptor (GR). Plants expressing any of these constructs were impaired in resistance to virulent and avirulent pathogens. The authors argued that the intermediate resistance exhibited in these lines indicates a function of cytosolic EDS1 for full resistance (García et al., 2010).

In the case of EDS1-NES and -GR lines, the possibility of residual nuclear EDS1 accumulation cannot be excluded which might cause the intermediate resistance phenotype. García et al. (2010) observed nuclear localization of the EDS1-YFP-NES protein in 5 % of Arabidopsis epidermal cells by imaging fluorescence in sections through individual nuclei. Around 20 % of isolated protoplast cells showed nucleo-cytoplasmic distribution of the EDS1 fusion protein. Proteins fused to GR are assumed to stay in the cytoplasm through their association with the HSP90 chaperone complex. However, there are indications that the complex might shuttle between cytosol and nucleus before Dex application (Echeverría et al., 2009). In this regard, it is conceivable that GR fusion does not completely deplete nuclear EDS1. Small amounts of the protein may enter the nucleus and might be sufficient to confer residual resistance. Evidence that very low levels of nucleo-cytoplasmic EDS1 are enough for partial resistance is provided by experiments using Col-*eds1* RNAi lines. These lines expressed strongly reduced amounts of EDS1 and still displayed partial resistance (Feys et al., 2005). Along the same line, marginal amounts of nuclear EDS1 in EDS1-NES or -GR lines might be responsible for the observed residual resistance.

However, there are also indications that EDS1-YFP-NLS protein might not be exclusively localized to the nucleus. Immunoblot analysis showed EDS1-YFP-NLS in the nuclei-depleted fraction (Figure 2.4). This might be due to leakiness of EDS1 from nuclei during biochemical fractionation as suggested by García et al. (2010) if EDS1 is not for example associated with chromatin or retained in higher order complexes. Alternatively, EDS1 might be bound to nuclear export components and therefore is present in the nuclei-depleted fraction. The amount of cytosolic EDS1 detected by this method is in clear contrast to the almost exclusive detection of EDS1-YFP-NLS in the nucleus by fluorescence imaging. Protein synthesis occurs in the cytoplasm. Therefore, at least a minor proportion of EDS1-YFP-NLS pool is located in this compartment until it is taken up by the nuclear pore trafficking machinery and transferred into the nucleus. This might be sufficient to induce EDS1 cytosolic signaling leading to resistance. There is no method available that restricts protein localization absolutely to one compartment and prevents it from shuttling at the same time. Hence, it cannot be finally concluded if presence of either EDS1-YFP-NES or -NLS or shuttling itself might affect the observed outputs in the analyzed lines.

Regarding defense outputs activated in plants with high enforced nuclear EDS1 accumulation (Figures 2.3, 2.7, 2.8), I assume a stronger effect of nuclear EDS1 for resistance. Additionally, all analyzed EDS1-YFP-NLS lines conferred wt-like resistance, whereas plants with EDS1 restricted to the cytoplasm showed intermediate resistance (Figure 2.19 and García et al. (2010)), supporting the hypothesis of nuclear EDS1 being the crucial factor for complete resistance.

Taking all data together, I favor a model in which nuclear EDS1 is necessary and sufficient to initiate defense responses such as transcriptional reprogramming and for bacterial growth containment. A balance of nucleo-cytoplasmic activity might be needed to control nuclear activity (see below). Results from previous work and this study indicate that plants can cope with massive amounts of EDS1 in both compartments and display wt-like development and resistance as in EDS1-YFP and EDS1-YFP-NLS #A5/wtEDS1 lines, as long as there is sufficient EDS1 in the nucleus (Figures 2.3, 2.4, 2.19, 2.31, 2.32).

3.2 High nuclear-restricted EDS1 accumulation induces defense responses in the absence of a pathogen stimulus

This study showed that plants with high EDS1 levels restricted to the nucleus show growth defects, defense-related transcriptional reprogramming and increased SA accumulation in the absence of a pathogen effector trigger (Figures 2.3, 2.5B, 2.6, 2.8). Together with the suppression of morphological and molecular changes at higher temperatures (Figure 2.11, 2.13), the exhibited features of EDS1-YFP-NLS #A5 and #A3 resemble constitutive activated resistance response since these are common features of autoimmune mutants (Bowling et al., 1994, 1997; Li et al., 2001; Yoshioka et al., 2001; Shirano et al., 2002; Yang and Hua, 2004).

Growth defects of plants with constitutive defense responses are thought to represent a trade-off between enhanced resistance and plant performance (Tian et al., 2003; Alcázar and Parker, 2011). Plants with continuously activated energy-costly defense responses lack resources to maintain regular development. It can be speculated that also in lines EDS1-YFP-NLS #A5 and # A3, morphological abnormalities are caused by the metabolic costs of activated defense signaling. In contrast to described autoimmunity mutants and the #A3 line which exhibit dwarfism from early development (Bowling et al., 1994, 1997; Li et al., 2001; Shirano et al., 2002), EDS1-YFP-NLS #A5 plants started to display growth defects after ~five weeks (Figure 2.3). This correlates with increasing amounts of EDS1 over time (Figure 2.16). I assume that a certain level of nuclear EDS1 protein needs to be passed to induce defense signaling and thereby growth defects.

EDS1 contributes to temperature sensitivity of immune responses even though it is unlikely to be a temperature sensor itself (Yang and Hua, 2004; Wang et al., 2009). Hence, the observation that high temperature suppresses EDS1-dependent defense outputs is not surprising. So far, the precise components of temperature sensing in plant immunity are not completely understood. There is a growing body of evidence pointing to NB-LRR proteins as major temperature sensors (Zhu et al., 2010). In the case of the EDS1-YFP-NLS lines, it can be hypothesized that other immune components than R proteins are the temperature sensing regulators since R proteins act upstream of the EDS1 signaling pathway (Zhang et al., 2003; Wirthmueller et al., 2007). Notably, dwarfism in plants induced by combined overexpression of *EDS1* and *PAD4* is temperature insensitive (Gobbato et al, manuscript in preparation). This indicates that a strong activation of EDS1 signaling together with PAD4 can override reduced activity of temperature sensors at high temperature.

Analysis of cell death response indicates a higher number of putative infection sites upon *H. arabidopsidis* inoculation in EDS1-YFP-NLS #A5 (Figure 2.10B), suggesting a reduced threshold for cell death initiation in this line. Based on the cell death assays of this study, it is not conclusive if the observed cell death is related to defense-induced HR. It might be caused by the shift to lower temperature after pathogen treatment since low temperature was shown to induce cell death in unchallenged EDS1-YFP-NLS #A5 plants (Figure 2.10A). This might result from amplified defense responses in EDS1-YFP-NLS # A5 at 19°C (Figure 2.14) or due to premature senescence accelerated at reduced temperature. Analysis of a common senescence marker gene, *SAG12*, which is thought to be specifically induced during developmental senescence (Gan and Amasino, 1997) was not significantly upregulated in EDS1-YFP-NLS #A5 plants. This suggests that cell death in EDS1-YFP-NLS #A5 is defense-related as also shown for other described lesions mutants with constitutive resistance (Bowling et al., 1997; Shirano et al., 2002). However, ion leakage experiments argue against a massive effector-triggered HR response in EDS1-YFP-NLS #A5 (Figure 2.9). To further analyze the impact of pathogen-induced cell death in EDS1-YFP-NLS lines, transgenic plants with enforced nuclear EDS1 accumulation can be generated in the Ws-0 background. Ws-0 plants display a stronger cell death response upon infection with *P. syringae* DC3000 expressing AvrRps4 compared to Col-0. Therefore, the window to monitor ion leakage between positive and negative control is broader and it will be easier to detect significant differences. Alternatively, it will be worth testing the *H. arabidopsidis* infection system using the available transgenic plants without temperature shift or including a water treatment control to be able to distinguish between temperature- and pathogen-induced cell death. This will be analyzed in the near future.

Induced defense outputs were specific for plants with high nuclear-restricted EDS1 levels and were not found in transgenic lines with high nuclear and additional cytoplasmic EDS1 or low amounts of EDS1-YFP-NLS (Figures 2.3, 2.7, 2.8). Analysis of three independent transgenic EDS1-YFP-NLS lines suggests that a certain threshold of EDS1 in the nucleus has to be reached before spontaneous defense activation occurs. Plants with EDS1-YFP-NLS levels similar to Col-0 behaved wt-like in all tested conditions. Plants with high EDS1-YFP-NLS amounts started to show defense outputs after ~five weeks, whereas plants with even stronger EDS1-YFP-NLS accumulation exhibited defense outputs already early in development (Figures 2.15, 2.16). I conclude that presence of nuclear EDS1 alone is insufficient to induce defense outputs without pathogen stimulus. A specific amount of EDS1-YFP-NLS has to accumulate before changes take place. Moreover, high nuclear EDS1 by itself (as in EDS-YFP plants) is not sufficient to stimulate defense responses; it has to be nuclear-restricted (Figure 2.3, 2.4, 2.8).

3.2.1 Induced defense outputs in EDS1-YFP-NLS cause marginally enhanced disease resistance

The observed induced defense responses in line EDS1-YFP-NLS #A5 suggest enhanced resistance as found in other mutants displaying constitutive activated defense (Bowling et al., 1994, 1997; Petersen et al., 2000; Li et al., 2001; Shirano et al., 2002). Surprisingly, there was no strongly elevated resistance detectable in EDS1-YFP-NLS #A5 towards the tested virulent and avirulent pathogens *H. arabidopsidis* Noco2, *P. syringae* DC3000 and *P. syringae* DC3000 AvrRps4 (Figure 2.19). In incompatible interactions, the EDS1-YFP-NLS #A5 resistance phenotype was not distinguishable from wt plants. Since wt plants are already resistant, it might be difficult to measure enhanced resistance. In this respect, it is more meaningful to study compatible interactions to analyze enhanced basal resistance compared to wt. Indeed, a trend of increased basal resistance in EDS1-YFP-NLS #A5 was statistically significant when all independent experiments were combined (Table 2.2). A plausible explanation for the small increase in resistance in contrast to the extent of defense activation in EDS1-YFP-NLS #A5 might be that EDS1 lies downstream in defense signaling compared to constitutive resistance due to autoactivated R proteins (Li et al., 2001; Frost et al., 2004; Maekawa et al., 2011). In these cases, activation of the more upstream positioned R proteins might induce additional signals leading to enhanced resistance. However, at least RPS4 signaling depends entirely on EDS1 (Wirthmueller et al., 2007), thereby arguing against this scenario.

More than 3700 genes were found to be differentially regulated in Ws-0 plants 6h after *P. syringae* DC3000 AvrRps4 infiltration (Bartsch et al., 2006). A subset of ~1200 of the AvrRps4-induced genes were not significantly changed in Col-0 EDS1-YFP-NLS #A5 plants (Figure 2.5B). According to GO-term analysis, most genes of this subset are related to defense (data not shown). These gene expression experiments were performed independently of each other using different Arabidopsis accessions. Therefore, they cannot be directly compared. However, I hypothesize that the sum of all genes reprogrammed during infection is required for a stronger resistance phenotype. Hence, line EDS1-YFP-NLS #A5 might miss a group of genes that are changed due to pathogen effector recognition during defense activation in wt plants, preventing enhanced basal resistance to the same extent as in described autoimmunity mutants (Bowling et al., 1994, 1997; Li et al., 2001; Zhou et al., 2004). Pathogen assays are in progress including published autoimmunity mutants with demonstrated enhanced basal resistance as positive control to assess differences in more detail. Most of my pathogen assays were performed before obvious morphological defects were detected. However, defense gene expression was frequently monitored before infection and confirmed to be induced. For some bacterial assays, plants were used already showing morphological abnormalities. They displayed no changes in resistance compared to plants without growth defects. This suggests that the time point for detecting elevated resistance was not missed.

In summary, EDS1-YFP-NLS #A5 displayed marginally increased basal resistance to virulent bacterial and oomycete pathogens, consistent with induced defense outputs in this line. Considering the amplitude of defense activation in line EDS1-YFP-NLS #A5, a stronger effect on resistance might have been expected. A possible scenario is that the observed defense responses might not be directly associated with stronger resistance, thereby indicating an uncoupling of defense outputs and resistance. To my knowledge, there is no other described mutant displaying constitutive defense activation without significantly enhanced basal resistance. Following the example of NPR1, where according to the current model, NPR1 needs to be continuously degraded to maintain its function as transcriptional co-activator, it might be that clearance of EDS1 from the nucleus is essential for its activity. Removing "used" EDS1 to allow activation of "fresh" EDS1 might be impaired in EDS1-YFP-NLS #A5. Clearance might be accomplished for example by cytosolic EDS1, which is not present EDS1-YFP-NLS #A5. Therefore, function of EDS1 may be saturated, preventing activation of stronger enhanced resistance.

3.2.2 Cytosolic EDS1 or EDS1 nucleo-cytoplasmic transport counterbalances nuclear EDS1 activity

An interesting observation is that EDS1-YFP-NLS #A5 plants completely lost induced defense outputs after the introduction of wt EDS1 which accumulates as a nucleo-cytoplasmic protein. This suggests a function of cytosolic EDS1 in antagonizing or counterbalancing nuclear EDS1 activity. García et al. (2010) showed an increase of nuclear EDS1 shortly after pathogen inoculation which precedes or coincides with EDS1-dependent defense gene induction. The short-term elevated EDS1 level might initiate defense responses. At later time points, nuclear EDS1 becomes equilibrated with the cytoplasmic pool (García et al., 2010). Taken together with the suppression of EDS1-YFP-NLS #A5 defense outputs by the presence of wt EDS1, I propose that during the process of defense activation, nuclear EDS1 initiates defense responses and at later time points its activity has to be restricted before it exaggerates and becomes harmful for the plant. Counterbalancing of nuclear EDS1 activity might be part of the function of cytosolic EDS1. Possible mechanisms to limit activity of EDS1 in the nucleus might be by selective degradation or by sequestering nuclear EDS1 in the cytoplasm.

Even though there is a strong imbalance between nuclear and cytoplasmic pools in line #A5/wtEDS1 (low amounts of cytoplasmic EDS1 compared to massive EDS1-YFP-NLS accumulation), wt EDS1 prevents excessive EDS1-YFP-NLS activity. This suggests that not a quantitative balance but a balance of protein activity of both pools is required for an appropriate immune response as well as development.

Alternatively, nucleo-cytoplasmic translocation of EDS1 might be crucial for proper immune signaling. Several studies revealed that shuttling between these compartments is necessary for defense regulation. It was shown that the transcriptional co-activator NPR1 is retained in the cytoplasm in uninduced cells. Upon pathogen challenge, NPR1 enters the nucleus and activates defense gene expression (Mou et al., 2003). In addition, components involved in R protein-mediated pathogen recognition were found to be redistributed during defense activation. Presence of the *Tobacco mosaic virus* helicase p50 releases the plant protein NRIP1 (N RECEPTOR-INTERACTING PROTEIN1) from the chloroplast into the cytoplasm and nucleus, where it then directly interacts with the tobacco TIR-NB-LRR protein N (Caplan et al., 2008). Also the Arabidopsis Cys protease RD19 (RESPONSIVE TO DEHYDRATION19), important for resistance mediated by the TIR-NB-LRR protein RRS1-R (RESISTANT TO RALSTONIA SOLANACEARUM 1-R), is relocalized from putative prevacuolar vesicles to the nucleus in the presence of the *Ralstonia solanacearum* effector PopP2 (Bernoux et al., 2008). A further hint for the importance of nucleo-cytoplasmic trafficking in plant immunity was illustrated by the characterization of MOS mutants. MOS (MODIFIERS OF SNC1) mutants were identified in a screen for suppressors of *snc1* autoimmunity (Zhang and Li, 2005). Mutations in *MOS3*, *6* and *7* abolished *snc1* constitutive resistance responses. *MOS3* is highly similar to the mammalian nucleoporin 96 and was shown to localize to the nuclear envelop (Zhang and Li, 2005). *MOS6* encodes importin $\alpha 3$ and *MOS7* is the plant homolog to the animal nucleoporin Nup88 (Palma et al., 2005; Cheng et al., 2009). Based on these studies, transport of immune regulators is assumed to be an essential mechanism to control defense signaling. Experiments demonstrating EDS1 transport via the nuclear pore trafficking machinery (García et al., 2010) as well as reduced levels of EDS1 detected in *mos7* (Cheng et al., 2009) provide evidence that the transfer of EDS1 between the subcellular compartments might contribute to control EDS1 activity. I hypothesize that EDS1 facilitates shuttling of transcriptional repressors or other immune signaling components which are important for proper regulation of immunity and prevention of detrimental defense activity.

A possible experiment to assess if shuttling of EDS1 has an intrinsic function would be by generating transgenic lines in the *eds1-2* background expressing EDS1-NLS and EDS1-NES together. Thereby, most of the shuttling should be prevented and effects upon pathogen treatment could be analyzed. However, also this experimental setup cannot exclude residual activity caused by small amounts of trafficking EDS1 protein since both pools will enter the respective other compartment to be picked up by the nuclear pore trafficking machinery and translocated according to their signal sequence.

I monitored a reduction of EDS1-YFP-NLS levels in line #A5/wtEDS (Figure 2.32). Gene silencing of *EDS1* due to crossing can be excluded by the fact that *EDS1* transcript levels were not altered in #A5/wtEDS1 compared to EDS1-YFP-NLS #A5 (Figure 2.33). It is tempting to speculate that cytosolic EDS1 removes EDS1-stabilizing components from the nucleus e.g. by sequestering them in the cytosol. Alternatively, cytosolic EDS1 might promote proteasome-mediated degradation of nuclear EDS1. Analysis using the proteasome inhibitor MG132 might shed light on whether EDS1-YFP-NLS #A5 protein is stabilized in the absence of cytosolic EDS1.

I cannot exclude the possibility that the threshold for auto-induce defense outputs might not be reached due to reduced EDS1-YFP-NLS accumulation in #A5/wtEDS plants. Comparative analysis including EDS1-YFP-NLS #A5 plants containing the *sid2* mutation, which also displayed decreased EDS1-YFP-NLS levels but still exhibited dwarfism, and #A5/wtEDS1 revealed similar amounts of EDS1-YFP-NLS protein in both lines (Figure 2.34). This suggests sufficient nuclear EDS1 accumulation in #A5/wtEDS1 plants for an induction of defense responses. There might be a fine threshold for the activation of defense outputs which is not passed in #A5/wtEDS1. However, this would be in contrast to the observed correlation between severity of growth defects and protein accumulation in the three analyzed EDS1-YFP-NLS lines (Figures 2.1, 2.3).

Since also wt EDS1 amounts are reduced in #A5/wtEDS1 plants, a further explanation might be a negative feedback loop for the self-regulation of EDS1 levels and thereby activity, which is only functional in the presence of cytoplasmic EDS1. This implies that cytosolic EDS1 is able to register nuclear EDS1 defense activities and determines the appropriate functional level needed. If necessary, cytosolic EDS1 might reduce overall EDS1 amounts, for example via promoting EDS1 degradation. However, EDS1-YFP-NLS does not necessarily over-accumulate, as shown for line EDS1-YFP-NLS #B2. It seems therefore that a lack of cytosolic EDS1 is only critical when nuclear EDS1 exceeds a threshold.

A possible reason why the same molecule can fulfill different or even opposing functions in the different subcellular compartments might be due to its associations with various partners. This is already indicated by analyzing the localization and function of EDS1-PAD4 and EDS1-SAG101 complexes (Feys et al., 2005; Rietz et al., 2011) and might be true for other yet unknown EDS1 associations. Also, EDS1 protein might become post-translationally modified depending on the presence of e.g. kinases causing functional changes. However, no post-transcriptional modification of EDS1 have been reported.

3.2.3 Putative functions of cytoplasmic EDS1

Further evidence points to a role of cytoplasmic EDS1 in immunity (García et al., 2010). First, EDS1-GR lines without Dex treatment showed discrete but expanded cell death upon infection with an avirulent *H. arabidopsidis* isolate (García et al., 2010), suggesting that cytosolic EDS1 is able to initiate but cannot contain cell death. Nuclear EDS1 was hypothesized to control restriction of cell death thereby avoiding potential destructive cellular events (García et al., 2010). Plants with nuclear-restricted EDS1 displayed deregulated cell death after infection (Figure 2.10B). This indicates also an ability of nuclear EDS1 to induce cell death. Based on these observations, I propose that both EDS1 pools on their own are sufficient to initiate programmed cell death but require the respective other pool to control cell death expansion to prevent detrimental effects. Transgenic lines expressing EDS1 restricted either to the nucleus or cytoplasm did not induce spontaneous cell death, suggesting that EDS1 mislocalization does not lead to general deregulation of cell death.

Second, EDS1 forms molecularly and spatially distinct complexes with its partners PAD4 and SAG101, consistent with different functions of EDS1 complexes in different cell compartments (Feys et al., 2005; Rietz et al., 2011). Furthermore, the maintenance of EDS1 in cytosol and nucleus throughout infection and development suggests a need for both pools. A connection between *EDS1* and cell death regulation has already been shown in studies of Rustérucci et al. (2001) by analyzing *LSD1*, a negative regulator of cell death. *Lsd1* plants displayed local HR upon infection but failed to restrict subsequent cell death at the margins of HR sites. Their data implicates a requirement of *EDS1* and *PAD4* during *lsd1*-dependent runaway cell death which is unrelated to local *R* gene-mediated HR.

Recent publications revealed an uncoupling of disease resistance and cell death initiation triggered by R proteins. Work of Heidrich et al. (2011) demonstrated a requirement of the bacterial effector AvrRps4 in the nucleus for defense gene expression and bacterial growth containment in the absence of cell death. On the other hand, AvrRps4 restricted to the cytosol was able to induce cell death but plants were compromised in disease resistance. In a further study, by analyzing functions of the barley CC-NB-LRR protein MLA10 in different subcellular compartments, it was demonstrated that MLA10 forced into the nucleus conferred resistance without triggering HR, whereas cytoplasmic localized MLA10 activated a strong cell death response in the presence of the respective effector protein $\mathrm{AVR_{A10}}$ (Bai et al., 2012). MLA10 restricted to the cytoplasm was unable to induce disease resistance. In case of the potato R protein Rx, it was shown that overexpression of Rx in the nucleus was not able to induce HR and leaded to impaired resistance (Tameling et al., 2010). In contrast, high cytosolic levels of Rx resulted in HR and enhanced resistance without pathogen effector trigger. Mislocalization was achieved by altering activity of Ran GTPase-activating protein 2 (RanGAP2) which is involved in protein import into the nucleus (Tameling and Baulcombe, 2007; Tameling et al., 2010). Hence, activation of programmed cell death by defense components localized in the cytoplasm might be a common scheme and is not necessarily decisive for pathogen containment in resistance. This might explain increased susceptibility of EDS1-GR lines in the presence of activated cell death response (García et al., 2010).

The described examples of cytoplasmic processes required for cell death comprise pathogen effectors and plant R proteins. Along the same line, it is possible that cytoplasmic EDS1 is part of a complex with other cytoplasmically localized cell death regulators and thereby contributes to cell death initiation and/or propagation. This hypothesis is difficult to test using the transgenic lines at hand. EDS1-YFP-NES plants displayed hyphal growth upon infection with *H. arabidopsidis* Emwa1 (recognized by RPP4) but did not exhibit spreading cell death, which is in contrast to the described results of the EDS1-GR lines without Dex treatment upon infection (García et al., 2010). Since it is unknown in which subcellular compartment RPP4 or the recognized effector are active, interpretation of these results have to be taken with care.

Furthermore, suppression of defense outputs in EDS1-YFP-NLS #A5 through the presence of wt EDS1 suggests an antagonizing function of cytosolic EDS1 on nuclear activity, as discussed above. Therefore, a role of cytoplasmic EDS1 in modulating nuclear EDS1 signaling is likely.

3.3 EDS1 nuclear activity requires PAD4 and is independent of SAG101

I have demonstrated a requirement for *PAD4* in the induction of EDS1-YFP-NLS #A5 defense outputs based on the finding that loss of *PAD4* in EDS1-YFP-NLS #A5 plants abolished growth defects and activated defense responses (Figures 2.27, 2.28). By contrast, influence of *SAG101* on EDS1-YFP-NLS #A5 phenotypes was not detected. Analysis of lines with nuclear-restricted EDS1 in the *pad4*/*sag101* single or double mutant backgrounds suggests a requirement for nuclear EDS1 and PAD4 in TIR-NB-LRR-mediated resistance (Figure 2.29). Regarding the EDS1 levels in #A5/*pad4* and #A5/*pad4*/*sag101* lines, there is higher EDS1-YFP-NLS protein accumulation compared to #B2 (Figures 2.1, 2.30). Since line #B2 conferred wt-like resistance towards *P. syringae* DC3000 AvrRps4 in contrast to the observed susceptibility in #A5/*pad4* and #A5/*pad4*/*sag101* (Figures 2.19 A, 2.29), I conclude that impaired resistance is not due to insufficient amounts of EDS1-YFP-NLS protein in #A5/*pad4* and #A5/*pad4*/*sag101*. A similar argumentation was followed by Feys et al. (2005). They demonstrated by analyzing Col-*eds1*RNAi lines that low amounts of EDS1 in *pad4*/*sag101* mutants cannot be the sole reason for the observed hypersusceptibilty towards avirulent pathogens. Overall, susceptibility of *pad4* and *pad4*/*sag101* in the EDS1-YFP-NLS #A5 background is consistent with the impact of *PAD4* and *SAG101* on *EDS1* signaling in wt plants. Also, in *sag101* mutants, no differences in resistance compared to wt were detected (Feys et al., 2005). A role of *SAG101* in resistance was only registered in *pad4*/*sag101* double mutant. Contribution of *SAG101* is likely masked in *sag101* single mutants due to partial redundancy of *PAD4* and *SAG101*. Since SAG101 is found only in the nucleus whereas PAD4 is localized to the cytosol and nucleus, Feys et al. (2005) speculated that the observed increased susceptibility only in *pad4* single mutants is due to a cytosolic function of PAD4 which cannot be accomplished by SAG101.

Consistent with the findings of decreased wt EDS1 accumulation in *pad4* (Feys et al., 2005), EDS1-YFP-NLS protein levels were significantly reduced in the *pad4-1* mutant background (Figure 2.30). *Pad4-1*/*sag101-1* double mutants showed a trend of even lower EDS1-YFP-NLS accumulation. This result emphasizes the combined stabilizing function of PAD4 and SAG101 on EDS1. However, the described impact of *sag101* on wt EDS1 protein stability (63 % EDS1 in *sag101*) (Feys et al., 2005) is not reflected in the EDS1-YFP-NLS #A5 background (Figure 2.30). This suggests that high EDS1 accumulation outweighs the missing stabilizing effect of *sag101-1*. In agreement with the measured protein levels, transcriptional analysis showed that SAG101 also had no effect on *EDS1*-

YFP-NLS transcript accumulation (Figures 2.28, 2.30). Reduced EDS1-YFP-NLS protein amounts in plants lacking *PAD4* was already reflected at the transcript level (Figure 2.28). This is consistent with data indicating a role of EDS1/PAD4 complexes for a transcriptional feed forward loop (Zhou et al., 1998; Feys et al., 2001; Rietz et al., 2011).

Moreover, activated defense in the EDS1-YFP-NLS #A5 background did not compensate combined loss of *PAD4* and *SAG101* (Figure 2.29), indicating an intrinsic signaling function of *PAD4* and/or *SAG101*. Due to SAG101 exclusive presence in the nucleus, I reasoned that in case there is any contribution of *SAG101* to resistance non-redundant of *PAD4*, it might be detectable in EDS1-YFP-NLS #A5 lines with only nuclear EDS1 activity. Nevertheless, no specific effects were monitored in #A5/*sag101-1*.

Rietz et al. (2011) demonstrated different functions of EDS1 in complex with or dissociated from PAD4 in compatible and incompatible interactions. Since there are no cytosolic EDS1/PAD4 complexes in EDS1-YFP-NLS lines, I conclude that joined activity of EDS1 and PAD4 in the nucleus is sufficient for the analyzed resistance responses. For future experiments, it will be interesting to study if mislocalization of EDS1 affects PAD4 subcellular distribution. A straightforward experiment to test this would be by protoplast isolation of EDS1-YFP-NLS #A5 plant tissue and transient expression of PAD4-CFP followed by fluorescent microscopy analysis. Moreover, fusion of NLS to the EDS1 variant incapable of interacting with PAD4 (eds1L262P, Rietz et al. (2011)) would provide further insight into the requirement of direct nuclear EDS1/PAD4 interaction in defense signaling.

3.4 Induced defense outputs of EDS1-YFP-NLS plants are largely independent of the SA pathway

An important part of EDS1 defense signaling is to induce the SA pathway (Feys et al., 2001; Wiermer et al., 2005). EDS1 acts upstream of SA accumulation, illustrated by the finding that *eds1* allows SA-triggered activation of EDS1-dependent genes (Zhou et al., 1998; Falk et al., 1999; Feys et al., 2001). The fact that SA also activates *EDS1* gene expression itself points to a positive feedback loop, likely involved in defense potentiation (Wiermer et al., 2005; Vlot et al., 2009).

Therefore, I was curious to investigate the impact of SA signaling on EDS1-YFP-NLS #A5 phenotypes. Intriguingly, by crossing EDS1-YFP-NLS #A5 with mutants of key components of SA biosynthesis (*sid2-1*) and signaling (*npr1-1*), no major contribution of the SA pathway to EDS1-YFP-NLS #A5 phenotypes could be detected (Figures 2.20, 2.24). Previous studies already revealed an SA-independent pathway in EDS1-conditioned resistance (Bartsch et al., 2006). One well-characterized EDS1-activated and SA-independent gene is *FMO1* (Bartsch et al., 2006; Mishina and Zeier, 2006). *FMO1* is also induced in EDS1-YFP-NLS #A5 (Table 2.1), suggesting a stimulation of SA-independent defense responses in EDS1-YFP-NLS #A5 plants. This is further supported by the observation that out of seven identified genes (including *FMO1*) which are induced in an EDS1- and PAD4-dependent manner independently of SA (Bartsch et al., 2006), six are significantly upregulated in EDS1-YFP-NLS #A5 plants (data not shown).

Interestingly, small differences in the severity of developmental changes in #A5/*sid2-1* plants compared to EDS1-YFP-NLS #A5 were detected. Growth defects in #A5/*sid2-1* were slightly delayed (Figure 2.20A). This suggests a minor contribution of SA-triggered defense independent of *NPR1* signaling since #A5/*npr1-1* plants were indistinguishable from EDS1-YFP-NLS #A5 (Figure 2.24). This result is consistent with the finding that NPR1 works downstream of SA accumulation (Cao et al., 1994; Delaney et al., 1995). It is known that SA induces several *NPR1*-independent defense responses, for example camalexin synthesis or expression of a subset of defense genes (Zhao and Last, 1996; Jirage et al., 1999; Blanco et al., 2005). Also, analysis of mutants expressing constitutive resistance revealed several NPR1-independent defense responses (Bowling et al., 1997; Clarke et al., 1998; Yoshioka et al., 2001).

Considering that the EDS1-YFP-NLS #A5 defense outputs are largely independent of *ICS1* and *NPR1*, it will be interesting to investigate resistance phenotypes of #A5/*sid2-1* and #A5/*npr1-1* upon virulent and avirulent pathogen inoculation compared to *sid2-1* and *npr1-1* single mutants. Previous analysis showed an impairment of basal resistance in *sid2* and *npr1* (Bowling et al., 1997; Nawrath and Métraux, 1999). *Npr1* plants supported limited pathogen growth in EDS1-conditioned *R* gene-mediated resistance (Delaney et al., 1995) whereas *sid2* plants displayed extended HR and trailing necrosis after infection with the avirulent *H. arabidopsidis* isolates Cala2 in RPP2-conditioned resistance (Bartsch, 2005).

3.5 A short period of EDS1 nuclear accumulation might be insufficient for resistance

Given the fact that nuclear EDS1 is required for early induction of defense genes upon pathogen challenge, I aimed to determine the immediate effects of short-term nuclear-restricted EDS1 accumulation for resistance and compare them to defense outputs caused by prolonged enforced nuclear EDS1. Conditional nuclear EDS1 accumulation would also avoid side effects caused by high long-term accumulation as observed in line EDS1-YFP-NLS #A5.

Strikingly, plants expressing EDS1-YFP-NLS protein after estradiol treatment were as susceptible as *eds1-2* upon avirulent *P. syringae* infection (Figure 2.35). Several time periods of repetitive estradiol application were tested (up to 5 days) before plants were inoculated with pathogens; estradiol-inducible EDS1-YFP-NLS lines always showed hypersusceptibility (Figure 2.38). EDS1-YFP-nls plants exhibited minor but significant decreased susceptibility after estradiol application compared to the mock control (Figure 2.35). However, I anticipated full complementation of the estradiol-inducible EDS1-YFP-NLS/nls lines.

There are several possible explanations why the estradiol-inducible EDS1-YFP-NLS lines displayed hypersusceptibility. I excluded the possibility that the construct is not functional by resequencing the vector insert. Also, YFP signal was detected by immunoblot analysis and in confocal imaging after estradiol-treatment. Additional, the construct is derived from the same vector backbone that was used for generating functional pEDS1:EDS1-YFP-NLS plants. Therefore, a short period of nuclear-restricted EDS1 accumulation might not be sufficient for resistance. Since estradiol-inducible EDS1-YFP-nls lines unexpectedly did not behave wt-like, we have generated another control expressing EDS1-YFP without additional nuclear localization signal driven by the estradiol-inducible promoter.

If estradiol-inducible EDS1-YFP plants produce properly localized EDS1-YFP protein and still display hypersusceptibility, this would point to a requirement for prolonged EDS1 presence in the nucleus to confer resistance. One possibility may be that EDS1 is involved in chromatin remodeling, thereby preparing the DNA for future defense gene expression. Since plants lack adaptive immunity, there is a need for a sophisticated innate immune system coping with rapidly evolving pathogens. A possible strategy might be to adjust the immune system to the existing microbes and increase recognition specificity over generations by natural selection. Thereby, plants would become specialized in perceiving particular pathogens. Even though they would be able to respond effectively and rapidly to these pathogens, they would lose the capability to react to newly evolved pathogens. Another possible strategy would involve a less precise but more flexible recognition mechanism. This might be achieved by DNA modification during the plants life cycle rather than adaptations by natural selection to be able to react faster to changed conditions (Pecinka and Mittelsten Scheid, 2012; Dowen et al., 2012). For the second strategy, it might be advantageous not to fix changes into the genome. Instead, necessary DNA modifications might be obtained by chromatin remodeling or histone modification. Both strategies are not mutually exclusive. One function of EDS1 might be to be involved in chromatin remodeling or histone modifications and thereby preparing plants for pathogen attacks. This might explain the hypersusceptible phenotype of plants with only short exposure to nuclear EDS1.

If estradiol-induced EDS1-YFP plants exhibit wt-like resistance, we can conclude that short-term nuclear-restricted accumulation of EDS1 is not sufficient for resistance but needs additional activity of the cytoplasmic pool. Consistent with this, García et al. (2010) demonstrated that short-term nuclear localization of EDS1-GR after Dex treatment was sufficient to induce full resistance and defense-associated gene expression. This would argue against a direct role of nuclear EDS1 in chromatin remodeling.

A further possible explanation for the observed susceptibility of estradiol-inducible EDS1-YFP-NLS/nls might be that expression of protein is mainly limited to leaf veins and stomata upon estradiol spray application, as indicated by fluorescence microscopy analysis (Figure 2.40). Therefore, the majority of mesophyll and epidermis cells may lack EDS1 activity. However, this does not square with the residual resistance in estradiol-inducible EDS1-YFP-nls lines.

3.6 Nuclear EDS1 protein associations

The aim of this experiment was to identify new nuclear associations of EDS1 by exploiting the advantages provided by high nuclear EDS1 expression in EDS1-YFP-NLS #A5 plants. I hypothesize that EDS1 interacts with components involved in gene expression regulation since a direct association of EDS1 itself with the chromatin was not shown so far (S. Blanvillain-Baufumé, R.P. Huibers, unpublished data). EDS1 interacts with its signaling partners PAD4 and SAG101 (Feys et al., 2001, 2005) and resides in complexes with several TIR-NB-LRR proteins (Heidrich et al., 2011; Bhattacharjee et al., 2011; Kim et al., 2012). Possible associations with e.g. transcription factors are expected to be transient and therefore might be hard to detect. Hence, using gel-free mass spectrometry analysis on EDS1-YFP-NLS tissue extracts coupled to a highly sensitive Orbitrap mass analyzer (Hu et al., 2005; Roe and Griffin, 2006) was considered a viable approach to identify *in vivo* interactors of nuclear EDS1.

One interesting candidate interactor identified in this study is RPN2. RPN2 belongs to the 19S regulatory subunit of the 26S proteasome. In plants, most of the proteasome subunits lack functional identification. Few mutants are documented with specific or shared phenotypes in development and stress-responses (Yao et al., 2012). There is a growing body of evidence pointing to a crucial role of 26S proteasome-mediated degradation in innate immunity in Arabidopsis. One well-characterized example is the dual role of proteasome-mediated degradation of NPR1 that is needed in uninduced cells to prevent gene expression but also stimulates NPR1 activity upon SAR activation (Spoel et al., 2009). Further studies demonstrated a link between regulation of defense components and the proteasome, including the control of PRR and NB-LRR receptors (Goritschnig et al., 2007; Lu et al., 2011). Studies in yeast and metozoans revealed a direct involvement of components of the proteasome in gene transcription regulation. Subunits of the proteasome were shown to regulate transcription via proteolytic and non-proteolytic activities as recruitment of transcriptional co-activators, transcriptional elongation or ubiquitin-dependent histone modifications (Kwak et al., 2011). However, activity of the proteasome contributes to the regulation of numerous plant pathways (Vierstra, 2009). Therefore, impairment of its function might cause pleiotropic effects. Nevertheless, there are several studies showing a specific role of components of the 26S proteasome in Arabidopsis resistance, as revealed for RPN1a (Yao et al., 2012).

Moreover, RPN2 was also detected in an independent experiment using transgenic lines overexpressing EDS1 combined with estradiol-inducible PAD4 expression (H. Cui, personal communication). Search for interactors in this work was performed using a gel-based approach coupled to Q-TOF mass spectrometry analysis. Independent identification of RPN2 as an EDS1 interactor prompts a deeper analysis of its functional relevance in EDS1 resistance signaling.

A further interesting candidate identified is EIF4A1 (EUKARYOTIC TRANSLATION INITIATION FACTOR 4A1). There is no known function of EIF4A1 in Arabidopsis. Its annotation is based on sequence homology to animal EIF4A1. EIF4A1 belongs to the DEAD box helicase family (www.uniprot.org). In human, it is described to have ATP-dependent RNA helicase function. As part of the eIF4F complex, it is required for mRNA binding to ribosomes (Loh et al., 2009; Chang et al., 2009). Gene translation initiation is an important step in regulating protein activity. If EDS1 might be involved in this step, it opens a new field of potential EDS1 function at the post-translational level. Arabidopsis EIF4A1 is predicted to be nucleo-cytoplasmic localized. It can be hypothesized that EDS1 contributes to EIF4A1 translocation from the nucleus into the cytoplasm where it delivers mRNA encoding defense components to the ribosome.

The severe growth defects associated with defense induction of EDS1-YFP-NLS #A5 suggest a cooperation of EDS1 with proteins involved in development. However, none of the identified proteins show a direct link to plant development. Furthermore, known interactions of EDS1 with R proteins were not detected in this experiment. A plausible explanation may be very low abundance of these complexes. On the other hand, there might be the need for a pathogen stimulus leading to an association of R proteins with EDS1 as an activation of R proteins by effector recognition.

3.7 Integration of new insights of EDS1 function in resistance

Results of this study show that EDS1 in the nucleus is sufficient to confer wt-like resistance at least against the tested pathogens. Analyses of plants with excessive nuclear EDS1 suggest an even mildly increase in resistance in certain cases and a contribution of cytosolic EDS1 in balancing nuclear activity. I propose a model integrating the results of my work with previous knowledge, illustrated in Figure 3.1.

(1) Upon pathogen effector recognition, there is a rise of nuclear EDS1 to induce defense responses by orchestrating gene expression changes (García et al., 2010). This increase might be via enhanced transport of EDS1 into the nucleus, by stabilization via its partners PAD4 and SAG101 or through further unknown mechanisms such as preventing EDS1 from degradation or its association with the chromatin.

(2) During its transfer into the nucleus, EDS1 might facilitate co-transfer of transcription factors (TFs). Inside the nucleus, EDS1 builds transient higher order complexes, leading to dynamic transcription or repression of specific genes. So far, we have no evidence for EDS1 directly acting at the chromatin. Nevertheless, there is data indicating association of EDS1 with certain TFs (T. Griebel, R. P. Huibers, personal communication). Additionally, EDS1 might promote TFs activities at the chromatin and regulate gene expression by sequestering transcriptional repressors (TRs). EDS1-mediated transcriptional reprogramming includes amongst others induction of *ICS1*, leading to SA accumulation and thereby activation of the SA signaling pathway.

(3) There is evidence that nuclear in cooperation with cytosolic EDS1 regulates distinct programmed cell death. In view of the results obtained in this study together with previous data (García et al., 2010), both pools seem to be sufficient to trigger cell death. Based on

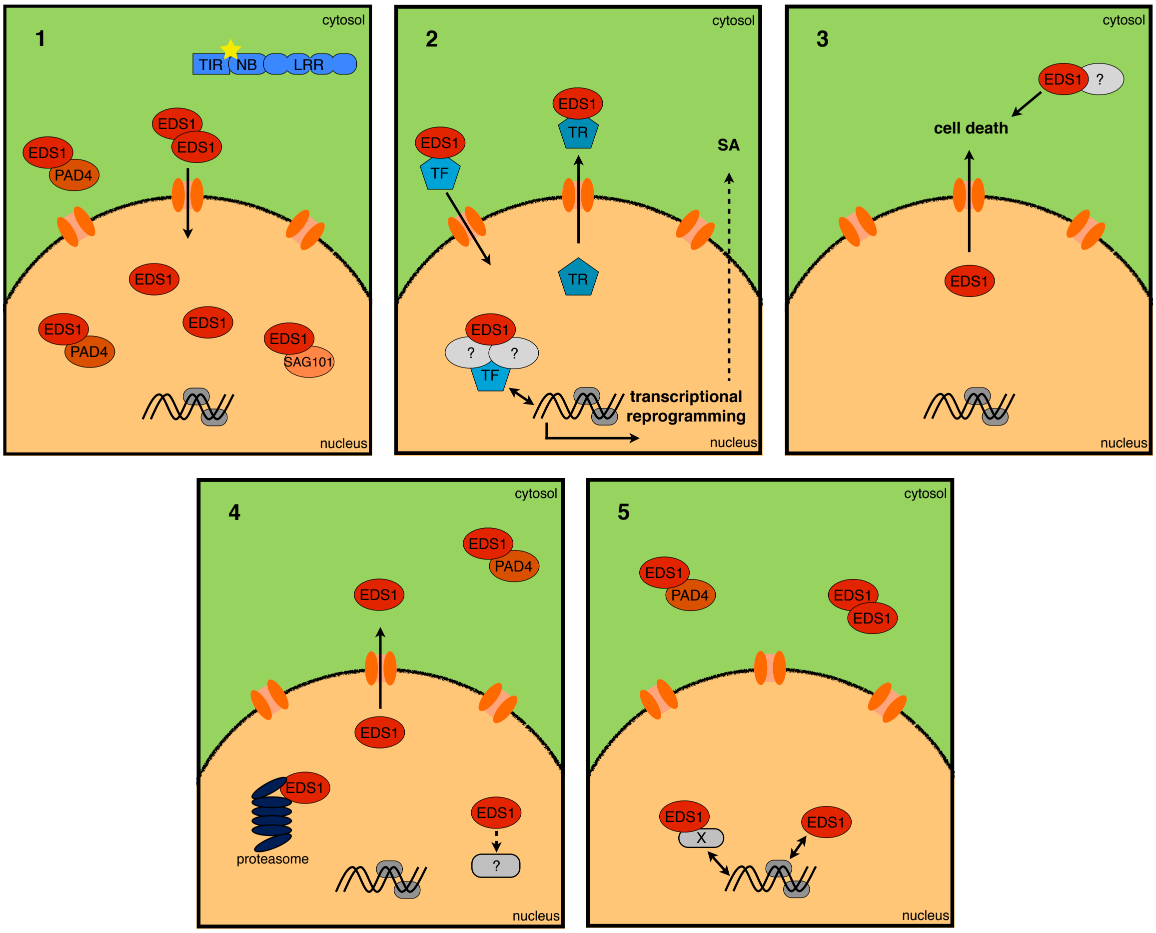

Figure 3.1: Models of various EDS1 functions during plant defense (1) Nuclear EDS1 level increases early upon TIR-NB-LRR activation potentially due to enhanced nuclear import or stabilization in the nucleus by its interaction partners. **(2)** EDS1 might control transcription factor (TF) and transcriptional repressor (TR) activity by regulating their localization. **(3)** Cytosolic and nuclear EDS1 functions are required for a balanced programmed cell death response. **(4)** Elevated nuclear EDS1 levels become equilibrated with the cytoplasmic pool. Possible mechanisms to reduce the nuclear EDS1 pool is by degradation via the proteasome, export into the cytosol or removal of stabilization components. **(5)** EDS1 might be involved in chromatin remodeling by interaction with chromatin remodeling proteins (X) to regulate histone (grey shapes) modifications. Nuclear EDS1-SAG101 complexes were not included in this scheme for simplicity.

analysis of EDS1-GR lines and plants with excessive nuclear EDS1 activity, the respective other pool appears to be involved in restricting programmed cell death, suggesting a requirement of balanced activity of cytosolic and nuclear EDS1 especially for this defense response.

(4) After the initial phase of launching defense signaling, EDS1 nuclear activity becomes reduced to prevent devastating effects for the plant. Increased EDS1 nuclear levels are equilibrated with the cytosolic pool. Plausible mechanisms to reduce nuclear EDS1 might be by removal of stabilizing components and enhanced export into the cytoplasm or increased proteasome-mediated degradation.

(5) Prolonged activity of nuclear EDS1 at the chromatin might be required for plants to confer full resistance. EDS1 might prepare DNA via chromatin modifications to "prime" plants for future pathogen attacks.

The most important finding of this study is that nuclear EDS1 activity by itself is sufficient to initiate defense responses such as cell death induction, defense gene expression and resistance to biotrophic and hemibiotrophic pathogens. My results indicate a requirement for an appropriate nucleo-cytoplasmic balance to fine tune EDS1 activity. Plants are able to tolerate variable and unbalanced EDS1 levels up to a threshold when it affects development and disease resistance. Interestingly, expression analysis of EDS1-YFP-NLS #A5 showed a discrepancy between moderate *EDS1* gene induction and high protein accumulation over time (Figures 2.15B, 2.16). This observation points to post-transcriptional stabilization of EDS1 and is consistent with the hypothesis mentioned above stating that cytosolic EDS1 might sequester stabilization components or induce proteasome-mediated degradation. An analog mechanism might be responsible for the early increase of nuclear EDS1 upon pathogen challenge, supported by the finding that nuclear protein enrichment preceded transcriptional EDS1 induction (García et al., 2010).

To deeper analyze if EDS1 more directly regulates transcriptional reprogramming during defense activation, EDS1-YFP-NLS #A5 lines can be used as a tool to test association of EDS1 with the chromatin. Previous attempts to identify potential EDS1 chromatin associations in an induced defense background by using the α-EDS1 antibody for protein pull-down did not reveal any significant interactions. High amounts of nuclear EDS1 in EDS1-YFP-NLS #A5 with age-induced defense responses might improve conditions to detect putative EDS1/DNA interaction. Additionally, given the fact that EDS1-NLS lines possess an YFP tag, α-GFP antibody can be used for chromatin-immunoprecipitation which is likely to be more effective in protein pull-down than the α-EDS1 antibody.

3.8 Future perspectives

The results presented in this study demonstrate that nuclear EDS1 is necessary and sufficient for the initiation of defense responses and to confer resistance to the tested pathogens. Overexpression of nuclear-restricted EDS1 resulted in induced defense signaling such as transcriptional reprogramming of defense-related genes in the absence of a pathogen stimulus. Results of experiments with estradiol-inducible EDS1-NLS lines indicated that there might be a need for continuous nuclear EDS1 accumulation to induce full resistance. It will be essential to test the currently generated estradiol-inducible EDS1-YFP control lines for their resistance phenotype. My results support previous indications of a more direct participation of EDS1 in chromatin regulation. To analyze if there is an association of EDS1 with chromatin is in my opinion the most important next step. Line EDS1-YFP-NLS #A5 represents an excellent tool to test this hypothesis. Five-week-old EDS1-YFP-NLS #A5 plants show uniformly activated defense responses including defense-related transcriptional reprogramming. Leaf tissues of these plants can be subjected to chromatin immunoprecipitation (ChIP) by using an α-GFP antibody which was shown to be highly efficient for this approach. ChIP will be followed by RT-qPCR on selected genes. Genes will be chosen based on existing knowledge in our lab obtained by gene expression microarray analysis and ChIP-sequencing data of the EDS1-dependent TIR-NB-LRR protein RPS4.

My results showed a dependency of nuclear EDS1 activity on PAD4. Given the fact that EDS1 and PAD4 stabilize each other, it would be worth testing whether the nucleo-cytoplasmic distribution of PAD4 in EDS1-YFP-NLS lines is affected by the nuclear restriction of EDS1. To gain deeper insight into the signaling functions of EDS1 and PAD4 in a complex or separated, an EDS1-NLS construct incapable of interacting with PAD4 could be characterized. Together with further structural analysis of the various EDS1/PAD4/SAG101 complexes, this will help to reveal the precise molecular functions of these proteins in the different subcellular compartments.

In addition, it will be interesting to investigate the resistance response triggered by an EDS1-dependent but non-nuclear TIR-NB-LRR protein in EDS1-YFP-NLS lines. This would address the question whether it is necessary for EDS1 to be present in the same compartment and potentially associate with R proteins for its signaling function. Overexpression of nuclear-restricted EDS1 triggered auto-induced defense responses. It will be worth examining whether induced defense in these plants results in a primed state for secondary infections. This can be analyzed in a classical SAR experiment.

To detect new components of the EDS1 signaling pathway, further investigations of EDS1 association with the newly identified putative interactors e.g. in yeast two-hybrid screens, by bimolecular fluorescence assays and later on *in planta* will be informative. The potential interaction with RPN2 and possibly also other subunits of the proteasome will be followed up since there is emerging evidence for a role of the proteasome in plant resistance (Yao et al., 2012). It will offer valuable insight to examine if EDS1 - as a master regulator of plant immunity - is also involved in this part of defense regulation. Moreover, EDS1-YFP-NLS #A3 plants, displaying defense responses and severe growth defects already at the seedling stage, are used in a suppressor screen to identify EDS1 genetic interactors.

4 Material and Methods

4.1 Material

4.1.1 Plant materials

Arabidopsis thaliana (here referred to as Arabidopsis) wild-type, mutant, and transgenic lines used for this study are listed in Table 4.1, 4.2 and 4.3, respectively.

Table 4.1: Wild-type Arabidopsis lines

Accession	Abbreviation	Original Source
Columbia	Col-0	J. Dangl[a]
Landsberg-*erecta*	L*er*-0	Nottingham *Arabidopsis* Stock Center[b]

[a] University of North Carolina, Chapel Hill, NC, USA, [b] Nottingham, UK

Table 4.2: Mutant Arabidopsis lines

Mutant allele	Accession	Mutagen	Reference/Source
eds1-2	Col-0 / (L*er*-0)[a]	FN	Bartsch et al. (2006)
pad4-1	Col-0	EMS	Glazebrook et al. (1997)
sag101-1	Col-0	dSpm	Feys et al. (2005)
pad4-1/sag101-1	Col-0	EMS/dSPM	Feys et al. (2005)
sid2-1	Col-0	EMS	Wildermuth et al. (2001b)
npr1-1	Col-0	EMS	Cao et al. (1994)

[a] L*er eds1-2* allele introgressed into Col-0 genetic background, 8th backcross generation
EMS: ethyl methane sulfonate; FN: fast neutron; dSpm: defective suppressor-mutator

Table 4.3: Transgenic Arabidopsis lines

Line	Accession	Construct	Reference/Source
EDS1-YFP-NLS/nls	Col-0	pEDS1::EDS1-YFP-NLS/nls	García (2009)
EDS1-YFP-NLS/*sid2-1*	Col-0	pEDS1::EDS1-YFP-NLS/nls	this study
EDS1-YFP-NLS/*npr1-1*	Col-0	pEDS1::EDS1-YFP-NLS/nls	this study
pED:EDS1-YFP-NLS/nls	Col-0	pER8::EDS1-YFP-NLS/nls	this study
EDS1-YFP	Col-0	pEDS1::EDS1-YFP	García (2009)
35S:YFP	Col-0	p35S::YFP-HA	García et al. (2010)
35S:RPS4	Col-0	p35S::RPS4-HA-Strep	Wirthmueller et al. (2007)
35S:RPS4/*eds1-2*	Col-0	p35S::RPS4-HA-Strep	Wirthmueller et al. (2007)

4.1.2 Pathogens

Arabidopsis plants were inoculated with the bacterial strain *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 harboring either the empty broad host range vector pVSP61 (Innes et al., 1993) or expressing the *P. syringae* pv. *pisi* effector AvrRps4 (Hinsch and Staskawicz, 1996). The *Pst* isolates were originally obtained from R. Innes (Indiana University, Bloomington, Indiana, USA). *Pseudomonas fluorescens* (*Pfo*) for cell death assays were obtained from J. Dangl (University of North Carolina, Chapel Hill, NC, USA).

Furthermore, pathogen assays were conducted with the oomycete pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) using isolate Emwa1 (Holub et al., 1995) and Noco2 (Parker et al., 1993). *Hpa* Emwa1 and Noco2 were derived from isolated conidia from single seedlings.

Table 4.4: Interaction between *Hyaloperonospora arabidopsidis* isolates and Arabidopsis ecotypes

Arabidopsis	*Hyaloperonospora arabidopsidis* isolate	
ecotype	**Emwa1**	**Noco2**
Col-0	incompatible (RPP4)	compatible
L*er*	incompatible (RPP4 and RPP8)	incompatible (RPP5)

4.1.3 Bacterial strains

Escherichia coli strains

For standard cloning the *Escherichia coli* strains DH10B and DH5α were used.

Agrobacterium tumefaciens strains

For stable transformation of Arabidopsis plants, DNA constructs were transformed in *Agrobacterium tumefaciens* strain GV3101 carrying the helper plasmid pMP90 (with resistance to Rifampicin and Gentamycin) or the helper plasmid pMP90RK (with resistance to Rifampicin, Kanamycin, and Gentamycin) (Koncz and Schell, 1986).

4.1.4 Media

Media were sterilized by autoclaving at 121°C for 20 min. The media was cooled before adding heat labile antibiotics.

Pseudomonas syringae media:

<u>NYG broth</u>	5	g/l	Peptone
(Nutrient-Yeast-Glycerol)	3	g/l	Yeast extract
	20	ml/l	Glycerol
			pH 7

For NYG agar plates, 5 g/l Bactoagar (Bacton, Franklin Lakes, USA) was added to NYG broth.

Arabidopsis thaliana media:

0.5 MS	4.9	g/l	MS powder including vitamins
(Murashige and Skoog)	5	g/l	Saccharose
	9	g/l	Plant agar
			pH 5.8 (KOH)

4.1.5 Buffers and solutions

Buffer and solution contents are listed in the following table. Buffers and solutions not displayed in this list are denoted with the corresponding methods. All buffers and solutions were prepared using Milli-Q water. Buffers and solutions for molecular biological experiments were autoclaved or sterilized using filter sterilization units.

DEPC-H_2O	Diethylpyrocarbonate	0.1 % in H_2O shake vigorously o/n, autoclave for 30 min
DNA extraction buffer	200 mM	Tris-Cl (pH 7.5)
	250 mM	NaCl
	25 mM	EDTA
	0.5 %	SDS
DNA gel loading dye	4 g	Sucrose
(6x)	100 mM	EDTA
	25 mg	Bromphenol blue
		dH_2O to 10 ml
Ponceau S	Ponceau S working solution was prepared by dilution of ATX Ponceau S concentrate (Fluka) 1:5 in H_2O	
SDS-PAGE running buffer	250 mM	Tris
(10x)	1.92 M	Glycine
	1 %	SDS

Protein sample buffer	0.125 M	Tris
(2x)	4 %	SDS
	20 %	Glycerol
	0.02 %	Bromphenol blue
	0.2 M	Dithiothreitol (DTT)
		pH 6.8

TAE buffer	242 g	Tris
(50x)	18.6 g	EDTA
	57.1 ml	Glacial acetic acid
		dH$_2$O to 1000 ml
		pH 8.5

TBS-T buffer	100 mM	TrisHCl
(10x)	1.5 M	NaCl
	0.05 %	Tween
		pH 8.0

Western blot transfer buffer	250 mM	Glycine
(10x)	500 mM	Tris
	12,5 ml	SDS (10 %)
		dH$_2$O to 1000 ml
		pH 9.2
		dilute 100 ml 10x buffer with 700 ml dH$_2$O and add 200 ml methanol

PCR buffer	500 mM	KCl
(10x)	100 mM	Tris HCl pH 9
	15 mM	MgCl$_2$
	1 %	Triton-X 100

Co-IP wash buffer	50 mM	Tris pH 8
	5 mM	EDTA
	150 mM	NaCl
	5 mM	DTT
	0.05 %	Triton-X 100

4.1.6 Chemicals and consumption items

Laboratory grade chemicals and reagents were purchased from the following companies unless otherwise stated:

- Gibo^{TM} BRL® (Neu Isenburg, Germany)
- Invitrogen^{TM}(Karlsruhe, Germany)
- Merck (Darmstadt, Germany)
- Roth (Karlsruhe, Germany)
- Serva (Heidelberg, Germany)
- Sigma-Aldrich (Deisenhofen, Germany)

The source of all kits is described in the sections where they are mentioned.

4.1.7 Antibiotics

Antibiotics were used in the concentrations listed below.

Table 4.6: Antibiotics stock solutions

Antibiotics	Concentration		
Ampicillin (Amp)	100	mg/l	in ddH_2O
Carbenicillin (Carb)	50	mg/l	in ddH_2O
Chloramphenicol	5	mg/l	in EtOH
Gentamycin (Gent)	25	mg/l	in ddH_2O
Kanamycin (Kan)	25	mg/l	in ddH_2O
Rifampicin (Rif)	100	mg/l	in DMSO
Spectinomycin (Spec)	100	mg/l	in ddH_2O
Tetracycline (Tet)	12.5	mg/l	in 50% EtOH

4.1.8 Oligonucleotides

The primers used in this study are listed in Table 4.7. Oligonucleotides were purchased from Invitrogen^{TM}(Karlsruhe, Germany), Metabion (Martinsried, Germany), Operon (Cologne, Germany), or Sigma-Aldrich (Deisenhofen, Germany). Lyophilized primers were resuspended in ddH_2O to a final concentration of 100 pmol/μl (=100 μM). Working solutions were diluted to 10 pmol/μl (=10 μM).

Table 4.7: Oligonucleotides used in this study

Primer	Purpose	Sequence (5‘-> 3‘)
	Genotyping	
R310	detection of npr1-1 mutation	TGAGTGCGGTTCTACCTTCC
R311	detection of npr1-1 mutation	ATGTCTCGAATGTACATAAGG
MS4	detection of sid2-1 mutation	GCAGTCCGAAAGACGACCTCGAG
MS5	detection of sid2-1 mutation	CTATCGAATGATTCTAGAAGAAGC
MW23	detection of eds1-2 mutation	CAAACGTCAAGAGAGCTGAG
EDS6	detection of eds1-2 mutation	GTGGAAACCAAATTTGACATTAG
105/E2	detection of eds1-2 mutation	ACACAAGGGTGATGCGAGACA
	qRT-PCR	
NP1	PR1 fw	TTCTTCCCTCGAAAGCTCAA
NP2	PR1 rev	AAGGCCCACCAGAGTGTATG
GP1	UBIQ fw	AGATCCAGGACAAGGAGGTATTC
GP2	UBIQ rev	CGCAGGACCAAGTGAAGAGTAG
GP3	EDS1 fw	CGAAGACACAGGGCCGTA
GP4	EDS1 rev	AAGCATGATCCGCACTCG
GP5	CBP60 fw	GGCGAGAAGTGAAGCTTTTG
GP6	CBP60 rev	GCGAAAATCCTTGACGGTTA
GP7	PBS3 fw	ACACCAGCCCTGATGAAGTC
GP8	PBS3 rev	CCCAAGTCTGTGACCCAGTT
GP11	PAD4 fw	GGTTCTGTTCGTCTGATGTTT
GP12	PAD4 rev	GTTCCTCGGTGTTTTGAGTT
GP13	SAG101 fw	CATTCCTCTGCTCCGAGAAC
GP14	SAG101 rev	CGTTTTAACGTCGGTTCGAT
GP15	PBS3 fw	ACACCAGCCCTGATGAAGTC
GP16	PBS3 rev	CCCAAGTCTGTGACCCAGTT
GP17	ISC1 fw	TTCTGGGCTCAAACACTAAAAC
GP18	ISC1 rev	GGCGTCTTGAAATCTCCATC

4.1.9 Enzymes

Restriction endonucleases

Restriction enzymes were purchased from New England Biolabs (Frankfurt, Germany). Enzymes were supplied with 10x reaction buffer which was used for restriction digests.

Nucleic acid modifying enzymes

Standard PCR and qRT-PCR reactions were performed using home-make *Taq* DNA polymerase. For cDNA synthesis, SuperScript^{TM}II RNase H-Reverse Transcriptase from Invitrogen^{TM}(Karlsruhe, Germany) was used.

4.1.10 Antibodies

Primary and secondary antibodies used for immunoblot detection are listed below. Primary antibody dilutions were prepared in TBS-T with the indicated milk concentration.

Table 4.8: Primary antibodies

Antibody	Source	Dilution	Reference
α-EDS1	rabbit polyclonal	1:500, 2% milk (w/v)	S.Rietz; J. Parker[a]
α-GFP	mouse monoclonal	1:2000	Roche (Mannheim, Germany)
α-Histone H3	rabbit polyclonal	1:500, 5% milk (w/v)	Abcam (Cambridge, UK)
α-PAD4	rabbit polyclonal	1:500	S.Rietz; J. Parker[a]
α-PEPC	rabbit polyclonal	1:500, 2% milk (w/v)	Rocklands, Gilbertsville, PA, USA

[a] Max Planck Institute for Plant Breeding Research, Cologne, Germany

Table 4.9: secondary antibodies

Antibody	Feature	Dilution	Source
goat anti-rabbit IgG-HRP	horseradish peroxidase conjugated	1:5000, 2% milk (w/v)	Santa Cruz (Santa Cruz, USA)
goat anti-mouse IgG-HRP	horseradish peroxidase conjugated	1:5000, 2% milk (w/v)	Santa Cruz (Santa Cruz, USA)

4.2 Methods

4.2.1 Growth conditions and maintenance of Arabidopsis plants

Seeds were sown on wet soil (Stender, Schermbeck, Germany) containing 10 mg 1-1 Confidor® WG 70 (Bayer, Germany) and first vernalized for 2 days at 4°C in the dark covered with a propagator lid to synchronize germination. Afterwards, the seeds were transferred to a plant growth chamber. Plants for experiments were grown at short day conditions (10h photoperiod, light intensity of app. 200 μEinsteins m^{-2} s^{-1}, 22°C and 60 % humidity). Three to five days post-germination, propagator lids were removed. For seed production, 3-week-old plants were transferred to long day conditions (16h photoperiod) and allowed to flower. To collect seeds, aerial plant part was enveloped in paper bags until siliques were ripe.

4.2.2 Generation of Arabidopsis F_1 and F_2 progeny

Individual flowers were emasculated by using fine tweezers and a magnifying-glass. To prevent self-pollination, only flowers that had well-developed stigma but immature stamen were used for crossing. Fresh pollen from 3 to 4 independent donor stamens was dabbed onto each single stigma. F_1 seeds were harvested from mature siliques and allowed to dry.

The obtained seeds were grown as described above and allowed to self-pollinate. Produced F_2 seeds were collected and stored.

4.2.3 Salicylic acid measurement

SA measurements was obtained of leaf material (100 to 200 mg fresh weight) according to Straus et al. (2010), using a chloroform/methanol extraction and analyzed by gas chromatography coupled to a mass spectrometer (GC-MS, Agilent, Santa Clare, USA).

4.2.4 Lactophenol trypan blue staining

Lactophenol trypan blue was used to visualize dead plant cells and *Hpa* hyphae (Koch and Slusarenko, 1990). Trypan blue stock solution was diluted with 1 volume of ethanol (95 %) before use. Leaves were placed into 15 ml Sarstedt tubes (Nümbrecht, Germany), covered with lactophenol trypan blue solution and boiled in a water bath for app. 1 min. For destaining, the solution was replaced twice with chloral hydrate (2.5 g/ml dH_2O) and incubated overnight on a rotor. Leaf material was left in 70 % glycerol for at least 2-3 hours before mounting onto glass microscope slide with 70 % glycerol and examined with an Axio Imager (Zeiss, Jena, Germany).

4.2.5 Pathogen maintenance and pathology assays

Pst and *Pfo* strains were streaked out on selective NYG agar plates from - 80°C DMSO or glycerol stocks. Streaked plates were incubated for 72 h at 28°C before re-streaked and kept for 48 h at 28°C. *Pst* inoculation was performed on 4 to 6 week-old plants if not noted otherwise, grown in pots with 5 plants per pot, by spray infection with a bacteria suspension of 1 x 10^7 cfu/ml in 10 mM $MgCl_2$ containing 0.04 % Silwet L-77 (Lehle seeds, Round Rock, USA). Bacterial titers were determined by shaking leaf discs at 28°C in 10 mM $MgCl_2$ supplemented with 0.001 % Silwet L-77 for 1 h (Tornero and Dangl, 2001; García et al., 2010). The resulting bacterial suspensions were serially diluted and spots of 20 μl per dilution were plated on selective NYG agar plates and incubated at 28°C for for 48 h before colonies were counted. For cell death assays, *Pfo* expressing AvrRps4 were syringe-infiltrated into leaves of 5-week-old plants. The bacteria concentration was adjusted to OD_{600}= 0.3 in 10 mM $MgCl_2$. After infiltration, leaf discs were washed in 30 ml H_2O for 30 min. Replicate samples of 3 leaf discs were transferred into in one well of a 24-well microtiter plate containing 2 ml H_2O. To determine ion leakage, the conductivity of 60 μl of each sample was measured with the conductivity meter Horiba Twin cond B-173 (Horiba, Japan). *Hpa* isolates were maintained as mass conidiosporangia cultures on leaves of their susceptible Arabidopsis genotype over a 7 day cycle. *Hpa* inoculations were done on 2-week-old plants by spray-infection with *Hpa* conidiospore suspension of 4 x 10^{4} spores/ml. To determine the number of conidiospores, replicate samples of 300 - 500 mg of infected leaf tissue were harvested at 7 days after inoculation, vortexed in water and counted in a Neubauer chamber under the light microscope.

4.2.6 Confocal laser scanning microscopy (CLSM)

Detailed analysis of intracellular fluorescence war performed by confocal laser scanning microscopy using a Leica TCS SPS AOBS (Leica, Wetzlar, Germany) based Axiovert microscope equipped with an Argon ion laser as an excitation source. YFP tagged proteins were excited by a 514 nm laser line. YFP fluorescence was selectively detected by using an HFT 514 dichroic mirror and BP 535-590 band pass emission filter. Images were analyzed with Leica Lite software.

4.2.7 Microarray analysis

Total RNA of 4-week-old plants was isolated with RNeasy Mini kit supplied with RNase-Free DNase set (QIAGEN) according to the manufacturer's instructions. RNA quality was assessed with RNA Nanochips on a Bioanalyzer (Agilent). Biotinylated cRNA was prepared according to a standard Ambion protocol from 1 μg total RNA (MessageAmp II-Biotin Enhanced Kit; Ambion). After amplification and fragmentation, 12.5 μg of cRNA was hybridized for 16 h at 45°C on GeneChip ATH1-121501 Genome Array. GeneChips were washed and stained with Fluidics Script FS450-004 in the Affymetrix Fluidics Station 450 and scanned using a GeneChip Scanner 3000 7G. The data were analyzed with Affymetrix GeneChip Operating Software version 1.4 using Affymetrix default analysis settings and global scaling as normalization method.

Probe signal values were subjected to the quantile normalization (Bolstad et al., 2003) and summarization using the GeneChip robust multi-array average (GC-RMA) algorithm (Wu and Irizarry, 2004) to obtain the expression level values of the genes. Results were analyzed by the following linear model using the lmFit function in the limma package in the R environment: log2 (expression level value) sample + replicate. The eBayes function in the limma package was used for variance shrinkage in calculation of the p-values. The Storey's q-values were calculated using the q-value function in the q-value package from the p-values (Storey and Tibshirani, 2003).

In order to select candidate genes, all genes that were at least two-fold differentially regulated in comparison to Col-0 and exhibited a p-value < 0.05 were used for the analysis. Analysis of overrepresented gene ontologies was carried out by using the online tool VirtualPlant 1.3 (http://virtualplant.bio.nyu.edu/cgi-bin/vpweb/) (Katari et al., 2010). Genevestigator V3 (https://www.genevestigator.com/gv/index.jsp) was used for meta-analysis of gene expression (Hruz et al., 2008).

4.2.8 Protein identification

Proteins of the eluate obtained from co-immunoprecipitation were purified by Filter Aided Sample Preparation (FASP) (J. Wisniewski, 2009) to remove glycerol and bromphenol blue. This method includes protein digestion. Samples were loaded onto a 10 cm (first replicate) or 15 cm (second and third replicates) by 75 microns C18 nano-LC column for reverse phase chromatography. Peptides were eluted into a LTQ Orbitrap Discovery mass spectrometer (Thermo Scientific, Waltham, USA) over an ESI interface. The Orbitrap detects all ions simultaneously over a given period of time thereby providing high resolution. Data processing was performed using the ProteinScape database system version 3.0 (Bruker Daltonics, Bremen), which initiated Mascot version 2.3 (Matrix Science) searches against a database including TAIR10, *Pseudomonas* and common artifacts sequences and additional revers decoys. For database searching, the delta M score for precursors and fragments was set to 0.01 Da and 0.5 Da, respectively. Only multiple charged peaks were collected and only one miss cleavage was tolerated. Oxidation (of Met) was allowed as a variable modification whereas carbamidomethylation (of Cys) was required. There is a 95% certainty threshold with a mascot score of 27. By this, the stringency was high enough so that no decoys survived. The delta M score for fragments was set to 0.5. At least 3 significant peaks for each peptide had to be detected before peptides were accepted for further analysis.

4.2.9 Molecular biological methods

4.2.9.1 Genomic DNA extraction

Genomic DNA was isolated in a quick procedure yielding in a small quantity of DNA of sufficient quality for PCR amplification. Leaf samples were taken by closing the cap of a 1.5 ml microcentrifuge tube onto a leaf to clip out a section of plant tissue. 400 μl of DNA extraction buffer were added and the tissue was ground with a micropestle. The solution was centrifuged at maximum speed for 5 min and 300 μl supernatant were transferred into a clean tube. To precipitate the DNA, 1 volume of isopropanol was added, the sample well mixed and then centrifuged at maximum speed for 5 min. The supernatant was discarded carefully and the remaining pellet rinsed with 750 μl of 70 % ethanol and dried on bench top. Finally, the pellet was dissolved in 100 μl H_2O and 3 μl were used for PCR.

4.2.9.2 RNA extraction

Total RNA was prepared from leaf tissue with the RNeasy® Plant Mini Kit from QIAGEN. Concentration and quality was determined using the NanoDrop photometer (PeqLab).

4.2.9.3 Polymerase chain reaction (PCR)

Standard PCR reactions were performed using home-made *Taq* Polymerase and were performed in a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad, Hercules, USA).

4.2.9.4 cDNA synthesis

SuperScriptII^{TM}RNase Reverse Transcriptase (Invitrogen^{TM}) was used for first strand cDNA synthesis by combining 2 μg template total RNA, 1 μl Oligo dT (500 μg/ml), 4 μl dNTP mix (each dNTP 2.5 mM) in a volume of 12 μl (deficit made up with DEPC-H_2O). To eliminate secondary structures, the sample was incubated for 5 min at 65°C before cooling on ice. For reverse transcription, 4 μl of 5x First-Strand Buffer (supplied with the enzyme), 2 μl of 0.1 M DTT and 0.5 μl reverse transcriptase was added to a final volume of 20 μl. The reaction was incubated at 42°C for 80 min before the enzyme was heat inactivated for 15 min at 70°C. The obtained cDNA solution was diluted 1:5 before using it for qRT-PCR.

4.2.9.5 Quantitative real time-PCR (qRT-PCR)

Quantitative RT-PCR experiments were performed on an iQ5 Real Time-PCR Detection System (Bio-Rad, Hercules, USA). Brilliant SYBR Green QPCR Core Reagent (Stratagen) or EvaGreen^{TM}(Biotium) was used as dye. The PCR amplification program is listed below. Relative transcript levels were calculated using the iQ5 Optical System Software (Version 2.0). *Ubitquitin* (At4g05320) transcript levels were used as an internal reference.

	Temperature	Time
Initial denaturation	95°C	3 min
Denaturation	95°C	10 sec
Annealing	60°C	30 sec
Extension	72°C	30 sec
	95°C	1 min
	55°C	1 min
Melting curve	55°C - 95°C	10 sec, 0.5°C steps, 81x
loop: denaturation - extension 40x		

RT-qPCR mix	
cDNA (1:5)	2 μl
dNTPs (2.5 mM each)	0.5 μl
10x PCR buffer	2.5 μl
Dye	1.25 μl
Glycerol (50 %)	4 μl
DMSO (100 %)	0.75 μl
Forward primer (10 μM)	0.5 μl
Reverse primer (10 μM)	0.5 μl
Taq polymerase	0.25 μl
dH_2O	add 25 μl

4.2.9.6 Agarose gel electrophoresis of DNA

DNA fragments were separated by by agarose gel electrophoresis in gels consisting of 1 - 2 % (w/v) agarose (Bio-Budget, Krefeld, Germany) supplied with ethidium bromide solution (2 μl/100 ml) in TAE buffer. 6x DNA loading buffer was added to the DNA samples before loading onto the agarose gel. Separated DNA fragments were visualized by placing the gel on a 312 nm UV transilluminator and photographed.

4.2.9.7 DNA sequencing and sequence analysis

DNA sequences were determined by the "Automatische DNA Isolierung und Sequenzierung" (ADIS) service unit at the MPIPZ on Applied Biosystems (Weiterstadt, Germany) Abi Prism 377 and 3700 sequencers using Big Dye-terminator chemistry. Sequence data was analyzed using SeqMan and SeqBuilder version 8.1 (DNASTAR, Madison, USA).

4.2.9.8 Plasmid DNA isolation from bacteria

Standard alkaline cell lysis minipreps of plasmid DNA were performed using the Macherey-Nagel plasmid prep kit according to the manufacturer´s instructions. Larger amounts of plasmid DNA were isolated using QIAGEN Midi preparation kit.

4.2.10 Biochemical methods

4.2.10.1 Total protein extraction

Total protein extracts were prepared from plant material (10 leaf discs, ø0.6 cm) frozen in liquid nitrogen. Samples were homogenized 2 x 15 sec to a fine powder using a mixer mill MM400 (Retsch, Haan, Germany) and 1.5 mm steel beads (Mühlmeier, Bärnau, Germany) in 1.5 tubes. 100 μl of 2x SDS-PAGE sample buffer was added to the samples on ice. Subsequently, samples were boiled for 10 min at 96°C while shaking at 300 rpm and, after chilling for 5 min on ice, centrifuged for 5 min at 4°C with maximum speed. If not directly loaded on a SDS gel, samples were stored at -20°C.

4.2.10.2 Co-immunoprecipitation from total plant extract

2 g leaf tissue of 5-week-old plants was ground in 2 ml Co-IP buffer supplemented with 1x proteinase inhibitor cocktail (= lysis buffer). The extract was centrifuged for 10 min at 4°C with maximum speed. Supernatant was filtered (d= 0.22 μm) and incubated with 50 μl anti-GFP MicroBeads (μMACS$^{\mathrm{TM}}$Epitope Tag Protein Isolation Kit, Miltenyi Biotec, Bergisch Gladbach, Germany) for 10 min on a rotor at 4°C and afterwards for 20 min on ice. The μcolumn was adjusted by applying 200 μl lysis buffer. After adding the lysate on the column, it was rinsed 5 times with 200 μl Co-IP buffer. Elution of the bound proteins was carried out with 50 μl pre-heated 95°C hot elution buffer (supplied by the kit). The eluate was pre-analyzed by SDS-PAGE and send to mass spectrometry.

4.2.10.3 Nuclear fractionation for immunoblot analysis

Nuclear fractionations were performed according to Feys et al. (2005). Two grams fresh weight of leaf tissue from 3- to 4-week-old plants were homogenized in 4 ml Honda buffer (2.5 % Ficoll 400, 5 % Dextran T40, 0.4 M Sucrose, 25 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$ and added before use: 5 mM DTT, 1 % protease inhibitor cocktail (SIGMA)) and then filtered through a 62 μm (pore size) nylon mesh. Triton X-100 was added to a final concentration of 0.5 %, mixed slowly and the mixture was incubated on ice for 15 min. Total fraction aliquot was taken at this point. The extract was centrifuged at 1500 g for 5 min and nuclei-depleted fraction aliquot was taken from the supernatant. The pellet was washed by gentle resuspension in 3 ml Honda buffer containing 0.1 % Triton X-100. The sample was centrifuged again at 1500 g for 5 min. The pellet was resuspended in 3 ml of Honda buffer and transferred to 1.5 ml microcentrifuge tubes. Starch and cell debris were removed by centrifugation at 100 g for 1 min. Supernatants were transferred to new microcentrifuge tubes and nuclei were pelleted by centrifugation at 2000 g for 5 min. Nuclear pellets were resuspended in 150 μl 2x SDS-PAGE loading buffer; this sample was called nuclei-enriched fraction. Total and nuclei-depleted fractions were mixed with 1 volume of 2x SDS-PAGE loading buffer and all samples were boiled for 8 min while shaking.

4.2.10.4 Denaturing SDS-polyacrylamide gel electrophoresis

Denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using Mini-PROTEAN 3 system (Bio-Rad, Hercules, USA). Tris-Glycine polyacrylamide (PAA) gels were prepared according to standard procedures (Sambrook et al., 2001), poured between two glass plates and overlaid with 1 ml isopropanol. After gels were polymerized, the alcohol overlay was removed and a stacking gel was poured on top of the resolving gel. A comb was inserted and the gel was allowed to polymerize. Resolving gels used in this study had a polyacrylamide content of 8 %, 10 %, or 15 % and stacking gels consisted of 5 % polyacrylamide. Gels prepared were of 1.5 mm thickness. After removing the comb, each PAA gel was placed into the electrophoresis tank and submerged in 1x running buffer. A pre-stained protein ladder (Thermo Scientific, Waltham, USA) and denatured protein samples were loaded on the gel and run at 90 V (stacking gel) and 120 V (resolving gel) until the desired separation was reached.

4.2.10.5 Immunoblot analysis

Proteins were electroblotted from the PAA gels to Hybond^{TM}-ECL^{TM}nitrocellusose membranes (Amersham Biosciences, Amersham, UK). PAA gels and membranes were pre-equilibrated in 1x transfer buffer. The blotting apparatus (Mini Trans-Blot® Cell, Bio-Rad) was assembled according to the manufacturer instructions. Transfer was carried out at 100 V for 70 min. Equal loading was determined by staining the membrane with Ponceau S for 5 min before rinsing the membrane. Destained membranes were blocked for 1 h at room temperature in TBS-T containing 5 % (w/v) non-fat dried milk powder (Roth). After blocking, membranes were incubated with primary antibodies over night (for 2 nights in case of α-EDS1) at 4°C while slowly shaking on a rotary shaker. Afterwards, membranes were washed 3 x 15 min with TBS-T and antibody-bound proteins were detected by incubating with secondary antibody solution for 1h at room temperature. Antibody solution was removed and membranes again washed 3 x 15 min with TBS-T. Detection of the protein of interest was carried out using the SuperSiganal® West Pico Chemiluminescence kit (Pierce, Thermo Scientific, Rockford, USA) or in a mixture of the SuperSiganal® West Pico Chemiluminescence kit and the SuperSiganal® West Femto Maximum Sensitivity kit according to the manufacturer instructions. Luminescence was detected by exposing the membranes to a photographic film (BioMax light film, Kodak).

Literature

Aarts, N., Metz, M., Holub, E., Staskawicz, B. J., Daniels, M. J. and Parker, J. E. (1998). Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signaling pathways in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 10306–11. ISSN 0027-8424.

Al-Quraan, N. a., Locy, R. D. and Singh, N. K. (2010). Expression of calmodulin genes in wild type and calmodulin mutants of Arabidopsis thaliana under heat stress. *Plant physiology and biochemistry : PPB / Société française de physiologie végétale* **48**, 697–702. ISSN 1873-2690.

Alcázar, R. and Parker, J. E. (2011). The impact of temperature on balancing immune responsiveness and growth in Arabidopsis. *Trends in plant science* , 1–10 ISSN 1878-4372.

Allan, W. L., Clark, S. M., Hoover, G. J. and Shelp, B. J. (2009). Role of plant glyoxylate reductases during stress: a hypothesis. *The Biochemical journal* **423**, 15–22. ISSN 1470-8728.

Amme, S., Matros, A., Schlesier, B. and Mock, H.-P. (2006). Proteome analysis of cold stress response in Arabidopsis thaliana using DIGE-technology. *Journal of experimental botany* **57**, 1537–46. ISSN 0022-0957.

Attaran, E., Zeier, T. E., Griebel, T. and Zeier, J. (2009). Methyl salicylate production and jasmonate signaling are not essential for systemic acquired resistance in Arabidopsis. *The Plant cell* **21**, 954–71. ISSN 1040-4651.

Ausubel, F. M. (2005). Are innate immune signaling pathways in plants and animals conserved? *Nature immunology* **6**, 973–9. ISSN 1529-2908.

Axtell, M. J. and Staskawicz, B. J. (2003). Initiation of RPS2-specified disease resistance in Arabidopsis is coupled to the AvrRpt2-directed elimination of RIN4. *Cell* **112**, 369–77. ISSN 0092-8674.

Bai, S., Liu, J., Chang, C., Zhang, L., Maekawa, T., Wang, Q., Xiao, W., Liu, Y., Chai, J., Takken, F. L. W., Schulze-Lefert, P. and Shen, Q.-H. (2012). Structure-function analysis of barley NLR immune receptor MLA10 reveals its cell compartment specific activity in cell death and disease resistance. *PLoS pathogens* **8**, e1002752. ISSN 1553-7374.

Bartsch, M. (2005). *Gene expression profiling leading to identification of essential components in EDS1/PAD4-regulated plant defence*. Ph.D. thesis.

Bartsch, M., Gobbato, E. and Bednarek, P. (2006). Salicylic acid-independent ENHANCED DISEASE SUSCEPTIBILITY1 signaling in Arabidopsis immunity and cell death is regulated by the monooxygenase FMO1. *The Plant cell* **18**, 1038–1051.

Bernoux, M., Timmers, T., Jauneau, A., Brière, C., de Wit, P. J. G. M., Marco, Y. and Deslandes, L. (2008). RD19, an Arabidopsis cysteine protease required for RRS1-R-mediated resistance, is relocalized to the nucleus by the Ralstonia solanacearum PopP2 effector. *The Plant cell* **20**, 2252–64. ISSN 1040-4651.

Bhattacharjee, S., Halane, M. K., Kim, S. H. and Gassmann, W. (2011). Pathogen Effectors Target Arabidopsis EDS1 and Alter Its Interactions with Immune Regulators. *Science* **334**, 1405–1408. ISSN 0036-8075.

Blanco, F., Garretón, V., Frey, N., Dominguez, C., Pérez-Acle, T., Van der Straeten, D., Jordana, X. and Holuigue, L. (2005). Identification of NPR1-dependent and independent genes early induced by salicylic acid treatment in Arabidopsis. *Plant molecular biology* **59**, 927–44. ISSN 0167-4412.

Bolstad, B. M., Irizarry, R. a., Astrand, M. and Speed, T. P. (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics (Oxford, England)* **19**, 185–93. ISSN 1367-4803.

Bostock, R. M. (2005). Signal crosstalk and induced resistance: straddling the line between cost and benefit. *Annual review of phytopathology* **43**, 545–80. ISSN 0066-4286.

Bowling, S., Clarke, J. and Liu, Y. (1997). The cpr5 mutant of Arabidopsis expresses both NPR1-dependent and NPR1-independent resistance. *The Plant cell* **9**, 1573–1584.

Bowling, S., Guo, A. and Cao, H. (1994). A mutation in Arabidopsis that leads to constitutive expression of systemic acquired resistance. *The Plant cell* .

Burch-Smith, T. M., Schiff, M., Caplan, J. L., Tsao, J., Czymmek, K. and Dinesh-Kumar, S. P. (2007). A novel role for the TIR domain in association with pathogen-derived elicitors. *PLoS biology* **5**, e68. ISSN 1545-7885.

Cao, H., Bowling, S. a., Gordon, a. S. and Dong, X. (1994). Characterization of an Arabidopsis Mutant That Is Nonresponsive to Inducers of Systemic Acquired Resistance. *The Plant cell* **6**, 1583–1592. ISSN 1532-298X.

Cao, H., Glazebrook, J., Clarke, J. D., Volko, S. and Dong, X. (1997). The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* **88**, 57–63. ISSN 0092-8674.

Caplan, J. L., Mamillapalli, P., Burch-Smith, T. M., Czymmek, K. and Dinesh-Kumar, S. P. (2008). Chloroplastic protein NRIP1 mediates innate immune receptor recognition of a viral effector. *Cell* **132**, 449–62. ISSN 1097-4172.

Century, K. S., Holub, E. B. and Staskawicz, B. J. (1995). NDR1, a locus of Arabidopsis thaliana that is required for disease resistance to both a bacterial and a fungal pathogen. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 6597–601. ISSN 0027-8424.

Chang, J. H., Cho, Y. H., Sohn, S. Y., Choi, J. M., Kim, A., Kim, Y. C., Jang, S. K. and Cho, Y. (2009). Crystal structure of the eIF4A-PDCD4 complex. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 3148–53. ISSN 1091-6490.

Cheng, Y. T., Germain, H., Wiermer, M., Bi, D., Xu, F., García, A. V., Wirthmueller, L., Després, C., Parker, J. E., Zhang, Y. and Li, X. (2009). Nuclear pore complex component MOS7/Nup88 is required for innate immunity and nuclear accumulation of defense regulators in Arabidopsis. *The Plant cell* **21**, 2503–16. ISSN 1040-4651.

Chisholm, S. T., Coaker, G., Day, B. and Staskawicz, B. J. (2006). Host-microbe interactions: shaping the evolution of the plant immune response. *Cell* **124**, 803–14. ISSN 0092-8674.

Chung, K. and Tasaka, M. (2011). RPT2a, a 26S proteasome AAA-ATPase, is directly involved in Arabidopsis CC-NBS-LRR protein uni-1D-induced signaling pathways. *Plant & cell physiology* **52**, 1657–64. ISSN 1471-9053.

Clarke, J. D., Aarts, N., Feys, B. J., Dong, X. and Parker, J. E. (2001). Constitutive disease resistance requires EDS1 in the Arabidopsis mutants cpr1 and cpr6 and is partially EDS1-dependent in cpr5. *The Plant journal : for cell and molecular biology* **26**, 409–20. ISSN 0960-7412.

Clarke, J. D., Liu, Y., Klessig, D. F. and Dong, X. (1998). Uncoupling PR gene expression from NPR1 and bacterial resistance: characterization of the dominant Arabidopsis cpr6-1 mutant. *The Plant cell* **10**, 557–69. ISSN 1040-4651.

Collier, S. M. and Moffett, P. (2009). NB-LRRs work a "bait and switch" on pathogens. *Trends in plant science* **14**, 521–9. ISSN 1878-4372.

Dangl, J. L. and Jones, J. D. G. (2001). Plant pathogens and integrated defence responses to infection. *Nature* **411**.

Delaney, T. P., Friedrich, L. and Ryals, J. a. (1995). Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resistance. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 6602–6. ISSN 0027-8424.

Dietrich, R. A., Richberg, M. H., Schmidt, R., Dean, C. and Dangl, J. L. (1997). A novel zinc finger protein is encoded by the Arabidopsis LSD1 gene and functions as a negative regulator of plant cell death. *Cell* **88**, 685–94. ISSN 0092-8674.

Dodds, P. N., Lawrence, G. J., Catanzariti, A.-M., Teh, T., Wang, C.-I. a., Ayliffe, M. a., Kobe, B. and Ellis, J. G. (2006). Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 8888–93. ISSN 0027-8424.

Dodds, P. N. and Rathjen, J. P. (2010). Plant immunity: towards an integrated view of plant-pathogen interactions. *Nature reviews. Genetics* **11**, 539–48. ISSN 1471-0064.

Dowen, R. H., Pelizzola, M., Schmitz, R. J., Lister, R., Dowen, J. M., Nery, J. R., Dixon, J. E. and Ecker, J. R. (2012). Widespread dynamic DNA methylation in response to biotic stress. *Proceedings of the National Academy of Sciences of the United States of America* **109**, E2183–91. ISSN 1091-6490.

Echeverría, P. C., Mazaira, G., Erlejman, A., Gomez-Sanchez, C., Piwien Pilipuk, G. and Galigniana, M. D. (2009). Nuclear import of the glucocorticoid receptor-hsp90 complex through the nuclear pore complex is mediated by its interaction with Nup62 and importin beta. *Molecular and cellular biology* **29**, 4788–97. ISSN 1098-5549.

Falk, a., Feys, B. J., Frost, L. N., Jones, J. D., Daniels, M. J. and Parker, J. E. (1999). EDS1, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 3292–7. ISSN 0027-8424.

Faria, A. M. C., Levay, A., Wang, Y., Kamphorst, A. O., Rosa, M. L. P., Nussenzveig, D. R., Balkan, W., Chook, Y. M., Levy, D. E. and Fontoura, B. M. a. (2006). The nucleoporin Nup96 is required for proper expression of interferon-regulated proteins and functions. *Immunity* **24**, 295–304. ISSN 1074-7613.

Feys, B., Wiermer, M., Bhat, R., Moisan, L., Medina-Escobar, N., Neu, C., Cabral, A. and Parker, J. (2005). Arabidopsis SENESCENCE-ASSOCIATED GENE101 stabilizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY1 complex in plant innate immunity. *The Plant cell* **17**, 2601–2613.

Feys, B. J., Moisan, L. J., Newman, M. a. and Parker, J. E. (2001). Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and PAD4. *The EMBO journal* **20**, 5400–11. ISSN 0261-4189.

Flor, H. (1971). Current status of the gene-for-gene concept. *Annual review of phytopathology* , 275–296.

Frost, D., Way, H., Howles, P., Luck, J., Manners, J., Hardham, A., Finnegan, J. and Ellis, J. (2004). Tobacco transgenic for the flax rust resistance gene L expresses allele-specific activation of defense responses. *Molecular plant-microbe interactions : MPMI* **17**, 224–32. ISSN 0894-0282.

Fu, Z., Yan, S., Saleh, A., Wang, W. and Ruble, J. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* , 1–6.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H. and Ryals, J. (1993). Requirement of salicylic Acid for the induction of systemic acquired resistance. *Science (New York, N.Y.)* **261**, 754–6. ISSN 0036-8075.

Gan, S. and Amasino, R. M. (1997). Making Sense of Senescence (Molecular Genetic Regulation and Manipulation of Leaf Senescence). *Plant physiology* **113**, 313–319. ISSN 1532-2548.

García, A. V. (2009). *Intracellular dynamics of Arabidopsis EDS1 immune regulatory complexes*. Ph.D. thesis.

García, A. V., Blanvillain-Baufumé, S., Huibers, R. P., Wiermer, M., Li, G., Gobbato, E., Rietz, S. and Parker, J. E. (2010). Balanced nuclear and cytoplasmic activities of EDS1 are required for a complete plant innate immune response. *PLoS pathogens* **6**, e1000970. ISSN 1553-7374.

Glazebrook, J., Zook, M., Mert, I. F., Kagan, I., Rogers, E. E., Crute, I. R., Holub, E. B., Hammerschmidt, R. and Ausubelt, F. M. (1997). Phytoalexin-Deficient Mutants of Arabidopsis Reveal That PAD4 Encodes a Regulatory Factor and That Four PAD Genes Contribute to Downy Mildew Resistance. *Genetics* **146**, 381–392.

Goff, S. a., Ricke, D., Lan, T.-H., Presting, G., Wang, R., Dunn, M., Glazebrook, J., Sessions, A., Oeller, P., Varma, H., Hadley, D., Hutchison, D., Martin, C., Katagiri, F., Lange, B. M., Moughamer, T., Xia, Y., Budworth, P., Zhong, J., Miguel, T., Paszkowski, U., Zhang, S., Colbert, M., Sun, W.-l., Chen, L., Cooper, B., Park, S., Wood, T. C., Mao, L., Quail, P., Wing, R., Dean, R., Yu, Y., Zharkikh, A., Shen, R., Sahasrabudhe, S., Thomas, A., Cannings, R., Gutin, A., Pruss, D., Reid, J., Tavtigian, S., Mitchell, J., Eldredge, G., Scholl, T., Miller, R. M., Bhatnagar, S., Adey, N., Rubano, T., Tusneem, N., Robinson, R., Feldhaus, J., Macalma, T., Oliphant, A. and Briggs, S. (2002). Genome-wide analysis of NBS-LRR-encoding genes in Arabidopsis. *Science* **296**, 92–100. ISSN 1095-9203.

Gómez-Gómez, L. and Boller, T. (2002). Flagellin perception: a paradigm for innate immunity. *Trends in plant science* **7**, 251–6. ISSN 1360-1385.

Goritschnig, S., Zhang, Y. and Li, X. (2007). The ubiquitin pathway is required for innate immunity in Arabidopsis. *The Plant journal : for cell and molecular biology* **49**, 540–51. ISSN 0960-7412.

Guo, L., Devaiah, S. P., Narasimhan, R., Pan, X., Zhang, Y., Zhang, W. and Wang, X. (2012). Cytosolic glyceraldehyde-3-phosphate dehydrogenases interact with phospholipase Dδ to transduce hydrogen peroxide signals in the Arabidopsis response to stress. *The Plant cell* **24**, 2200–12. ISSN 1532-298X.

Guo, M., Tian, F., Wamboldt, Y. and Alfano, J. R. (2009). The majority of the type III effector inventory of Pseudomonas syringae pv. tomato DC3000 can suppress plant immunity. *Molecular plant-microbe interactions : MPMI* **22**, 1069–80. ISSN 0894-0282.

Heidrich, K., Wirthmueller, L., Tasset, C., Pouzet, C., Deslandes, L. and Parker, J. E. (2011). Arabidopsis EDS1 Connects Pathogen Effector Recognition to Cell Compartment-Specific Immune Responses. *Science* **334**, 1401–1404. ISSN 0036-8075.

Hinsch, M. and Staskawicz, B. (1996). Identification of a new Arabidopsis disease resistance locus, RPS4, and cloning of the corresponding avirulence gene, avrRps4, from Pseudomonas syringae pv. pisi. *Molecular plant-microbe interactions : MPMI* **9**, 55–61. ISSN 0894-0282.

Hobbs, S. L., Kpodar, P. and DeLong, C. M. (1990). The effect of T-DNA copy number, position and methylation on reporter gene expression in tobacco transformants. *Plant molecular biology* **15**, 851–64. ISSN 0167-4412.

Holub, E. B., Brose, E., Tör, M., Clay, C., Crute, I. R. and Beynon, J. L. (1995). Phenotypic and genotypic variation in the interaction between Arabidopsis thaliana and Albugo candida. *Molecular plant-microbe interactions : MPMI* **8**, 916–28. ISSN 0894-0282.

Hruz, T., Laule, O., Szabo, G., Wessendorp, F., Bleuler, S., Oertle, L., Widmayer, P., Gruissem, W. and Zimmermann, P. (2008). Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. *Advances in bioinformatics* **2008**, 420747. ISSN 1687-8035.

Hu, Q., Noll, R. J., Li, H., Makarov, A., Hardman, M. and Graham Cooks, R. (2005). The Orbitrap: a new mass spectrometer. *Journal of mass spectrometry : JMS* **40**, 430–43. ISSN 1076-5174.

Hunt, M. D., Delaney, T. P., Dietrich, R. a., Weymann, K. B., Dangl, J. L. and Ryals, J. a. (1997). Salicylate-independent lesion formation in Arabidopsis lsd mutants. *Molecular plant-microbe interactions : MPMI* **10**, 531–6. ISSN 0894-0282.

Ichimura, K., Casais, C., Peck, S. C., Shinozaki, K. and Shirasu, K. (2006). MEKK1 is required for MPK4 activation and regulates tissue-specific and temperature-dependent cell death in Arabidopsis. *The Journal of biological chemistry* **281**, 36969–76. ISSN 0021-9258.

Innes, R. W., Bisgrove, S. R., Smith, N. M., Bent, a. F., Staskawicz, B. J. and Liu, Y. C. (1993). Identification of a disease resistance locus in Arabidopsis that is functionally homologous to the RPG1 locus of soybean. *The Plant journal : for cell and molecular biology* **4**, 813–20. ISSN 0960-7412.

J. Wisniewski, A. Zougman, N. N. M. M. (2009). Universal sample preparation method for proteome analysis. *Nature methods* **6**, 3–7.

Jirage, D., Tootle, T. L., Reuber, T. L., Frost, L. N., Feys, B. J., Parker, J. E., Ausubel, F. M. and Glazebrook, J. (1999). Arabidopsis thaliana PAD4 encodes a lipase-like gene that is important for salicylic acid signaling. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 13583–8. ISSN 0027-8424.

Jones, J. D. G. and Dangl, J. L. (2006). The plant immune system. *Nature* **444**, 323–9. ISSN 1476-4687.

Kalderon, D., Richardson, W., Markham, A. and Smith, A. (1984). Sequence requirements for nuclear location of simian virus 40 large-T antigen. *Nature* **311**, 33–38.

Katagiri, F. and Tsuda, K. (2010). Understanding the plant immune system. *Molecular plant-microbe interactions : MPMI* **23**, 1531–6. ISSN 0894-0282.

Katari, M. S., Nowicki, S. D., Aceituno, F. F., Nero, D., Kelfer, J., Thompson, L. P., Cabello, J. M., Davidson, R. S., Goldberg, A. P., Shasha, D. E., Coruzzi, G. M. and Gutiérrez, R. a. (2010). VirtualPlant: a software platform to support systems biology research. *Plant physiology* **152**, 500–15. ISSN 1532-2548.

Kim, M. G., da Cunha, L., McFall, A. J., Belkhadir, Y., DebRoy, S., Dangl, J. L. and Mackey, D. (2005). Two Pseudomonas syringae type III effectors inhibit RIN4-regulated basal defense in Arabidopsis. *Cell* **121**, 749–59. ISSN 0092-8674.

Kim, S. H., Gao, F., Bhattacharjee, S., Adiasor, J. a., Nam, J. C. and Gassmann, W. (2010). The Arabidopsis resistance-like gene SNC1 is activated by mutations in SRFR1 and contributes to resistance to the bacterial effector AvrRps4. *PLoS pathogens* **6**, e1001172. ISSN 1553-7374.

Kim, T.-H., Hauser, F., Ha, T., Xue, S., Böhmer, M., Nishimura, N., Munemasa, S., Hubbard, K., Peine, N., Lee, B.-H., Lee, S., Robert, N., Parker, J. E. and Schroeder, J. I. (2011). Chemical genetics reveals negative regulation of abscisic Acid signaling by a plant immune response pathway. *Current biology : CB* **21**, 990–7. ISSN 1879-0445.

Kim, T.-H., Kunz, H.-H., Bhattacharjee, S., Hauser, F., Park, J., Engineer, C., Liu, A., Ha, T., Parker, J. E., Gassmann, W. and Schroeder, J. I. (2012). Natural Variation in Small Molecule-Induced TIR-NB-LRR Signaling Induces Root Growth Arrest via EDS1- and PAD4-Complexed R Protein VICTR in Arabidopsis. *The Plant cell* **24**, 5177–5192. ISSN 1532-298X.

Koch, E. and Slusarenko, a. (1990). Arabidopsis is susceptible to infection by a downy mildew fungus. *The Plant cell* **2**, 437–45. ISSN 1040-4651.

Koncz, C. and Schell, J. (1986). The promoter of T,-DNA gene Scontrols the tissue-specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector .

Kwak, J., Workman, J. L. and Lee, D. (2011). The proteasome and its regulatory roles in gene expression. *Biochimica et biophysica acta* **1809**, 88–96. ISSN 0006-3002.

Laird, J., Armengaud, P., Giuntini, P., Laval, V. and Milner, J. J. (2004). Inappropriate annotation of a key defence marker in Arabidopsis: will the real PR-1 please stand up? *Planta* **219**, 1089–92. ISSN 0032-0935.

Li, X., Clarke, J. D., Zhang, Y. and Dong, X. (2001). Activation of an EDS1-mediated R-gene pathway in the snc1 mutant leads to constitutive, NPR1-independent pathogen resistance. *Molecular plant-microbe interactions : MPMI* **14**, 1131–9. ISSN 0894-0282.

Li, Y., Li, S., Bi, D., Cheng, Y. T., Li, X. and Zhang, Y. (2010). SRFR1 negatively regulates plant NB-LRR resistance protein accumulation to prevent autoimmunity. *PLoS pathogens* **6**, e1001111. ISSN 1553-7374.

Lipka, V., Dittgen, J., Bednarek, P., Bhat, R., Wiermer, M., Stein, M., Landtag, J., Brandt, W., Rosahl, S., Scheel, D., Llorente, F., Molina, A., Parker, J., Somerville, S. and Schulze-Lefert, P. (2005). Pre- and postinvasion defenses both contribute to nonhost resistance in Arabidopsis. *Science* **310**, 1180–3. ISSN 1095-9203.

Liu, J., Elmore, J. M., Lin, Z.-J. D. and Coaker, G. (2011). A receptor-like cytoplasmic kinase phosphorylates the host target RIN4, leading to the activation of a plant innate immune receptor. *Cell host & microbe* **9**, 137–46. ISSN 1934-6069.

Loh, P. G., Yang, H.-S., Walsh, M. a., Wang, Q., Wang, X., Cheng, Z., Liu, D. and Song, H. (2009). Structural basis for translational inhibition by the tumour suppressor Pdcd4. *The EMBO journal* **28**, 274–85. ISSN 1460-2075.

Louis, J., Gobbato, E., Mondal, H. a., Feys, B. J., Parker, J. E. and Shah, J. (2012). Discrimination of Arabidopsis PAD4 activities in defense against green peach aphid and pathogens. *Plant physiology* **158**, 1860–72. ISSN 1532-2548.

Lu, D., Lin, W., Gao, X., Wu, S. and Cheng, C. (2011). Direct ubiquitination of pattern recognition receptor FLS2 attenuates plant innate immunity. *Science* **332**, 1439–1442.

Mackey, D., Belkhadir, Y., Alonso, J. M., Ecker, J. R. and Dangl, J. L. (2003). Arabidopsis RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. *Cell* **112**, 379–89. ISSN 0092-8674.

Mackey, D., Holt, B. F., Wiig, A. and Dangl, J. L. (2002). RIN4 interacts with Pseudomonas syringae type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis. *Cell* **108**, 743–54. ISSN 0092-8674.

Maekawa, T., Cheng, W., Spiridon, L. N., Töller, A., Lukasik, E., Saijo, Y., Liu, P., Shen, Q.-H., Micluta, M. a., Somssich, I. E., Takken, F. L. W., Petrescu, A.-J., Chai, J. and Schulze-Lefert, P. (2011). Coiled-coil domain-dependent homodimerization of intracellular barley immune receptors defines a minimal functional module for triggering cell death. *Cell host & microbe* **9**, 187–99. ISSN 1934-6069.

Malamy, J., Carr, J., Klessig, D. and Raskin, I. (1990). Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. *Science* **250**, 1002–4. ISSN 0036-8075.

Mateo, A. and Mühlenbock, P. (2004). LESION SIMULATING DISEASE 1 is required for acclimation to conditions that promote excess excitation energy. *Plant Physiology* **136**, 2818–2830.

Mauch-Mani, B. and Slusarenko, a. J. (1996). Production of Salicylic Acid Precursors Is a Major Function of Phenylalanine Ammonia-Lyase in the Resistance of Arabidopsis to Peronospora parasitica. *The Plant cell* **8**, 203–212. ISSN 1532-298X.

Métraux, J., Signer, H. and Ryals, J. (1990). Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* **250**, 8–10.

Meyers, B., Kozik, A. and Griego, A. (2003). Genome-wide analysis of NBS-LRR-encoding genes in Arabidopsis. *The Plant cell* **15**, 809–834.

Mishina, T. and Zeier, J. (2006). The Arabidopsis flavin-dependent monooxygenase FMO1 is an essential component of biologically induced systemic acquired resistance. *Plant physiology* **141**, 1666–1675.

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H. and Shibuya, N. (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 19613–8. ISSN 1091-6490.

Moore, J. W., Loake, G. J. and Spoel, S. H. (2011). Transcription dynamics in plant immunity. *The Plant cell* **23**, 2809–20. ISSN 1532-298X.

Mou, Z., Fan, W. and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* **113**, 935–44. ISSN 0092-8674.

Mühlenbock, P., Szechynska-Hebda, M., Plaszczyca, M., Baudo, M., Mateo, A., Mullineaux, P. M., Parker, J. E., Karpinska, B. and Karpinski, S. (2008). Chloroplast signaling and LESION SIMULATING DISEASE1 regulate crosstalk between light acclimation and immunity in Arabidopsis. *The Plant cell* **20**, 2339–56. ISSN 1040-4651.

Nawrath, C. and Métraux, J. P. (1999). Salicylic acid induction-deficient mutants of Arabidopsis express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. *The Plant cell* **11**, 1393–404. ISSN 1040-4651.

Palma, K., Zhang, Y. and Li, X. (2005). An importin alpha homolog, MOS6, plays an important role in plant innate immunity. *Current biology* **15**, 1129–35. ISSN 0960-9822.

Parker, J., Holub, E. and Frost, L. (1996). Characterization of eds1, a mutation in Arabidopsis suppressing resistance to Peronospora parasitica specified by several different RPP genes. *The Plant cell* **8**, 2033–2046.

Parker, J. E., Szabo, V., Staskawicz, B. J., Lister, C., Dean, C., Daniels, M. J. and Jones, J. D. (1993). Phenotypic characterization and molecular mapping of the Arabidopsis thaliana locus RPP5, determining disease resistance to Peronospora parasitica. *The Plant journal* **4**, 821–831. ISSN 0960-7412.

Pecinka, A. and Mittelsten Scheid, O. (2012). Stress-induced chromatin changes: a critical view on their heritability. *Plant & cell physiology* **53**, 801–8. ISSN 1471-9053.

Pegadaraju, V., Knepper, C., Reese, J. and Shah, J. (2005). Premature leaf senescence modulated by the Arabidopsis PHYTOALEXIN DEFICIENT4 gene is associated with defense against the phloem-feeding green peach. *Plant Physiology* **139**, 1927–1934.

Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H. B., Lacy, M., Austin, M. J., Parker, J. E., Sharma, S. B., Klessig, D. F., Martienssen, R., Mattsson, O., Jensen, a. B. and Mundy, J. (2000). Arabidopsis map kinase 4 negatively regulates systemic acquired resistance. *Cell* **103**, 1111–20. ISSN 0092-8674.

Pieterse, C. M. J., Van der Does, D., Zamioudis, C., Leon-Reyes, A. and Van Wees, S. C. M. (2012). Hormonal modulation of plant immunity. *Annual review of cell and developmental biology* **28**, 489–521. ISSN 1530-8995.

Rietz, S., Stamm, A., Malonek, S., Wagner, S., Becker, D., Medina-Escobar, N., Vlot, a. C., Feys, B. J., Niefind, K. and Parker, J. E. (2011). Different roles of Enhanced Disease Susceptibility1 (EDS1) bound to and dissociated from Phytoalexin Deficient4 (PAD4) in Arabidopsis immunity. *The New phytologist* **191**, 107–19. ISSN 1469-8137.

Roe, M. R. and Griffin, T. J. (2006). Gel-free mass spectrometry-based high throughput proteomics: tools for studying biological response of proteins and proteomes. *Proteomics* **6**, 4678–87. ISSN 1615-9853.

Rustérucci, C., Aviv, D. H., Holt, B. F., Dangl, J. L. and Parker, J. E. (2001). The disease resistance signaling components EDS1 and PAD4 are essential regulators of the cell death pathway controlled by LSD1 in Arabidopsis. *The Plant cell* **13**, 2211–24. ISSN 1040-4651.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (2001). *Molecular Cloning: A Laboratory Manual*, volume 3 of *Molecular Cloning a laboratory manual.*. Cold Spring Harbor Laboratory Press. ISBN 0879695773.

Shen, Q.-H., Saijo, Y., Mauch, S., Biskup, C., Bieri, S., Keller, B., Seki, H., Ulker, B., Somssich, I. E. and Schulze-Lefert, P. (2007). Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses. *Science* **315**, 1098–103. ISSN 1095-9203.

Shirano, Y., Kachroo, P., Shah, J. and Klessig, D. (2002). A gain-of-function mutation in an Arabidopsis Toll Interleukin1 Receptor-Nucleotide Binding Site-Leucine-Rich Repeat type R gene triggers defense responses and results. *The Plant cell* **14**, 3149–3162.

Sohn, K. H., Zhang, Y. and Jones, J. D. G. (2009). The Pseudomonas syringae effector protein, AvrRPS4, requires in planta processing and the KRVY domain to function. *The Plant journal : for cell and molecular biology* **57**, 1079–91. ISSN 1365-313X.

Spoel, S. H., Mou, Z., Tada, Y., Spivey, N. W., Genschik, P. and Dong, X. (2009). Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. *Cell* **137**, 860–72. ISSN 1097-4172.

Storey, J. D. and Tibshirani, R. (2003). Statistical significance for genomewide studies. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 9440–5. ISSN 0027-8424.

Straus, M. R., Rietz, S., Ver Loren van Themaat, E., Bartsch, M. and Parker, J. E. (2010). Salicylic acid antagonism of EDS1-driven cell death is important for immune and oxidative stress responses in Arabidopsis. *The Plant journal : for cell and molecular biology* **62**, 628–40. ISSN 1365-313X.

Tameling, W. I. L. and Baulcombe, D. C. (2007). Physical association of the NB-LRR resistance protein Rx with a Ran GTPase-activating protein is required for extreme resistance to Potato virus X. *The Plant cell* **19**, 1682–94. ISSN 1040-4651.

Tameling, W. I. L., Nooijen, C., Ludwig, N., Boter, M., Slootweg, E., Goverse, A., Shirasu, K. and Joosten, M. H. a. J. (2010). RanGAP2 mediates nucleocytoplasmic partitioning of the NB-LRR immune receptor Rx in the Solanaceae, thereby dictating Rx function. *The Plant cell* **22**, 4176–94. ISSN 1532-298X.

Tian, D., Traw, M., Chen, J., Kreitman, M. and Bergelson, J. (2003). Fitness costs of R-gene-mediated resistance in Arabidopsis thaliana. *Nature* **423**, 74–77.

Tornero, P. and Dangl, J. L. (2001). A high-throughput method for quantifying growth of phytopathogenic bacteria in Arabidopsis thaliana. *The Plant journal : for cell and molecular biology* **28**, 475–81. ISSN 0960-7412.

Tsuda, K., Sato, M., Stoddard, T., Glazebrook, J. and Katagiri, F. (2009). Network properties of robust immunity in plants. *PLoS genetics* **5**, e1000772. ISSN 1553-7404.

Uv, A., Roth, P. and Xylourgidis, N. (2000). members only encodes a Drosophila nucleoporin required for Rel protein import and immune response activation. *Genes & Development* **14**, 1945–1957.

van der Hoorn, R. a. L. and Kamoun, S. (2008). From Guard to Decoy: a new model for perception of plant pathogen effectors. *The Plant cell* **20**, 2009–17. ISSN 1040-4651.

van Dijk, K., Tam, V. C., Records, A. R., Petnicki-Ocwieja, T. and Alfano, J. R. (2002). The ShcA protein is a molecular chaperone that assists in the secretion of the HopPsyA effector from the type III (Hrp) protein secretion system of Pseudomonas syringae. *Molecular microbiology* **44**, 1469–81. ISSN 0950-382X.

Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D. and Miyamoto, S. (1995). Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. *Genes & Development* **9**, 2723–2735. ISSN 0890-9369.

Vierstra, R. D. (2009). The ubiquitin-26S proteasome system at the nexus of plant biology. *Nature reviews. Molecular cell biology* **10**, 385–97. ISSN 1471-0080.

Vlot, a. C., Dempsey, D. A. and Klessig, D. F. (2009). Salicylic Acid, a multifaceted hormone to combat disease. *Annual review of phytopathology* **47**, 177–206. ISSN 0066-4286.

Wagner, S., Rietz, S., Parker, J. E. and Niefind, K. (2011). Crystallization and preliminary crystallographic analysis of Arabidopsis thaliana EDS1, a key component of plant immunity, in complex with its signalling partner SAG101. *Acta crystallographica. Section F, Structural biology and crystallization communications* **67**, 245–8. ISSN 1744-3091.

Wang, D., Amornsiripanitch, N. and Dong, X. (2006). A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. *PLoS pathogens* **2**, e123. ISSN 1553-7374.

Wang, Y., Bao, Z., Zhu, Y. and Hua, J. (2009). Analysis of temperature modulation of plant defense against biotrophic microbes. *Molecular plant-microbe interactions : MPMI* **22**, 498–506. ISSN 0894-0282.

Weiler, K. and Wakimoto, B. (1995). Heterochromatin and gene expression in Drosophila.

Wiermer, M., Cheng, Y. T., Imkampe, J., Li, M., Wang, D., Lipka, V. and Li, X. (2012). Putative members of the Arabidopsis Nup107-160 nuclear pore sub-complex contribute to pathogen defense. *The Plant journal : for cell and molecular biology* **70**, 796–808. ISSN 1365-313X.

Wiermer, M., Feys, B. J. and Parker, J. E. (2005). Plant immunity: the EDS1 regulatory node. *Current opinion in plant biology* **8**, 383–9. ISSN 1369-5266.

Wildermuth, M. C., Dewdney, J., Wu, G. and Ausubel, F. M. (2001a). Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* **414**, 562–5. ISSN 0028-0836.

Wildermuth, M. C., Dewdney, J., Wu, G. and Ausubel, F. M. (2001b). Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* **414**, 562–5. ISSN 0028-0836.

Wirthmueller, L., Zhang, Y., Jones, J. D. G. and Parker, J. E. (2007). Nuclear accumulation of the Arabidopsis immune receptor RPS4 is necessary for triggering EDS1-dependent defense. *Current biology* **17**, 2023–9. ISSN 0960-9822.

Wu, Z. and Irizarry, R. (2004). Preprocessing of oligonucleotide array data. *Nature biotechnology* **22**, 656–657.

Yang, S. and Hua, J. (2004). A haplotype-specific Resistance gene regulated by BONZAI1 mediates temperaturea-dependent growth control in Arabidopsis. *The Plant cell* **16**, 1060–1071.

Yao, C., Wu, Y., Nie, H. and Tang, D. (2012). RPN1a, a 26S proteasome subunit, is required for innate immunity in Arabidopsis. *The Plant journal : for cell and molecular biology* **71**, 1015–28. ISSN 1365-313X.

Yoshioka, K., Kachroo, P., Tsui, F., Sharma, S. B., Shah, J. and Klessig, D. F. (2001). Environmentally sensitive, SA-dependent defense responses in the cpr22 mutant of Arabidopsis. *The Plant journal : for cell and molecular biology* **26**, 447–59. ISSN 0960-7412.

Zhang, Y., Cheng, Y. T., Bi, D., Palma, K. and Li, X. (2005). MOS2, a protein containing G-patch and KOW motifs, is essential for innate immunity in Arabidopsis thaliana. *Current biology : CB* **15**, 1936–42. ISSN 0960-9822.

Zhang, Y., Fan, W., Kinkema, M., Li, X. and Dong, X. (1999). Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 6523–8. ISSN 0027-8424.

Zhang, Y., Goritschnig, S., Dong, X. and Li, X. (2003). A gain-of-function mutation in a plant disease resistance gene leads to constitutive activation of downstream signal transduction pathways in suppressor of npr1-1, constitutive 1. *The Plant cell* **15**, 2636–2646.

Zhang, Y. and Li, X. (2005). A putative nucleoporin 96 is required for both basal defense and constitutive resistance responses mediated by suppressor of npr1-1, constitutive 1. *The Plant cell* **17**, 1306–1316.

Zhao, J. and Last, R. L. (1996). Coordinate regulation of the tryptophan biosynthetic pathway and indolic phytoalexin accumulation in Arabidopsis. *The Plant cell* **8**, 2235–44. ISSN 1040-4651.

Zhou, F., Menke, F. L. H., Yoshioka, K., Moder, W., Shirano, Y. and Klessig, D. F. (2004). High humidity suppresses ssi4-mediated cell death and disease resistance upstream of MAP kinase activation, H2O2 production and defense gene expression. *The Plant journal : for cell and molecular biology* **39**, 920–32. ISSN 0960-7412.

Zhou, J., Trifa, Y. and Silva, H. (2000). NPR1 differentially interacts with members of the TGA/OBF family of transcription factors that bind an element of the PR-1 gene required for induction by salicylic acid. *Molecular plant-microbe interactions* **13**, 191–202. ISSN 0894-0282.

Zhou, N., Tootle, T. L., Tsui, F., Klessig, D. F. and Glazebrook, J. (1998). PAD4 functions upstream from salicylic acid to control defense responses in Arabidopsis. *The Plant cell* **10**, 1021–30. ISSN 1040-4651.

Zhu, S., Jeong, R. and Venugopal, S. (2011). SAG101 forms a ternary complex with EDS1 and PAD4 and is required for resistance signaling against turnip crinkle virus. *PLoS pathogens* **7**.

Zhu, Y., Qian, W. and Hua, J. (2010). Temperature modulates plant defense responses through NB-LRR proteins. *PLoS pathogens* **6**, e1000844. ISSN 1553-7374.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J. D. G., Boller, T. and Felix, G. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. *Cell* **125**, 749–60. ISSN 0092-8674.

Zuo, J., Niu, Q. and Chua, N. (2001). An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. *The Plant journal* **24**, 265–273.

5 Supplementary data

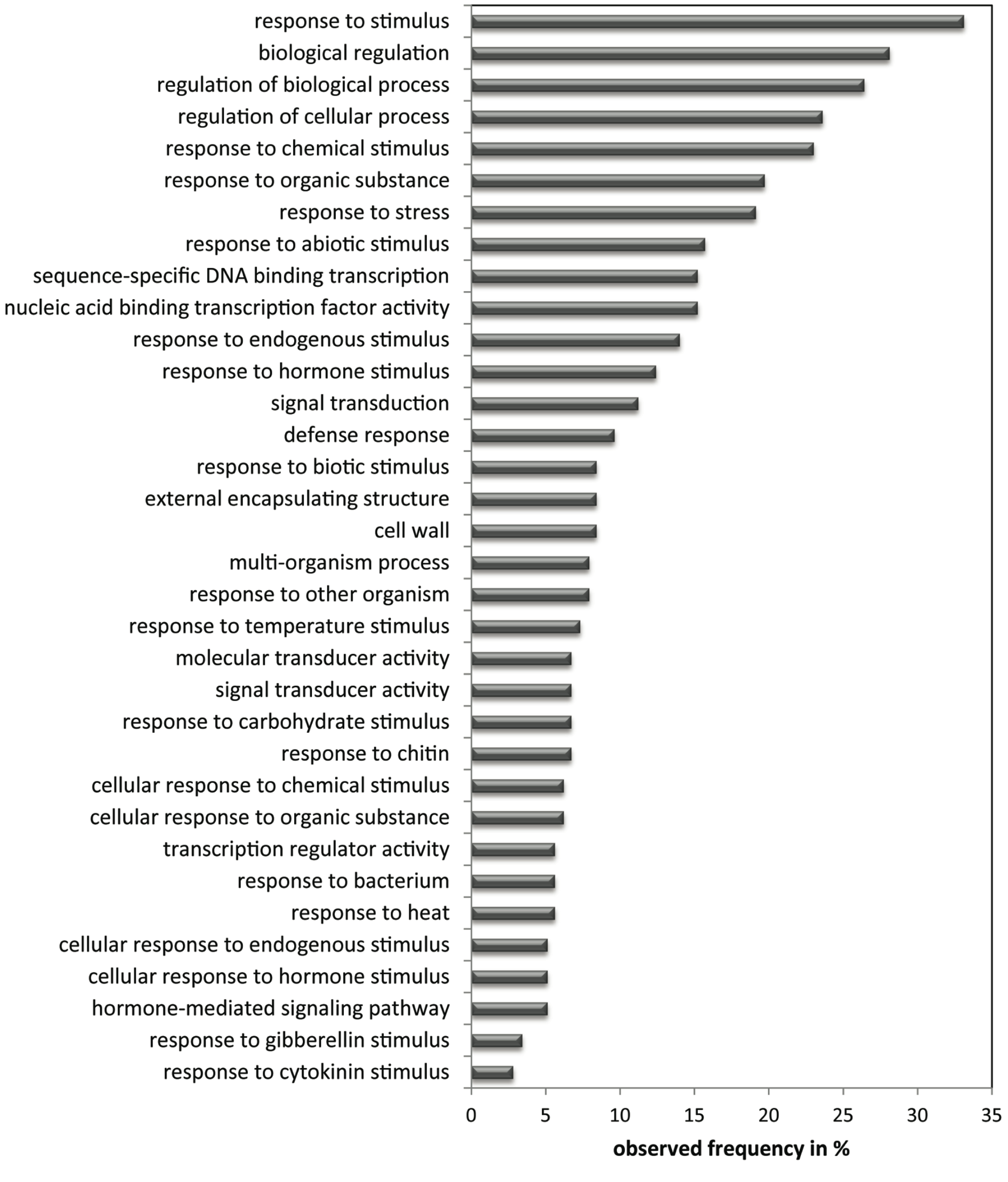

Figure 5.1: Transcriptional reprogramming in EDS1-YFP-nls plants. Gene Ontology (GO) term enrichment analysis of all differentially regulated genes with at least 2-fold changes and p-value < 0.01 in EDS1-YFP-nls compared to Col-0. GO terms were clustered into functional groups. GO term analysis (GO biological process) was performed using the platform VirtualPlant 1.3 (Katari et al., 2010); p-values were calculated with the Fisher Exact Test (with FDR correction); p-value cutoff was set to 0.01; Arabidopsis thaliana Columbia tair10 ATH1 was used as background population.

Acknowledgements

I would like to thank my supervisor Prof. Dr. Jane Parker for her valuable advice and her support during this work.

Furthermore, I want to thank Prof. Dr. Paul Schulze-Lefert for the possibility to work in his department at the MPIPZ and for accepting to evaluate my PhD thesis.

I also want to thank Prof. Dr. Ute Höcker and Dr. Laurent Deslandes for evaluating my thesis and participating in my thesis committee.
Thanks to the head of the thesis committee, Prof. Dr. Martin Hülskamp.

Thanks to Elmon and Tom for their technical support.

Many thanks to all past and present members of the JP group: Steffen, Ana, Johannes, Servane, Dieter, Jaqueline (thank you for all your help in the lab!), Thomas, Haitao, Friederike, Stephan, Anne and Marcel for your help and the nice atmosphere at work! It was a great pleasure working with you and spending so much time together during the last years!

Special thanks go to Katharina, Nico and the members of my IMPRS class!

Am allermeisten möchte ich mich bei meiner Familie bedanken: meine Eltern, Linda und Julian. Ihr habt mich immer unterstützt und ich weiß, auf euch kann ich mich verlassen. Vielen Dank für dafür!

Declaration

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Paul Schulze-Lefert betreut worden.

Köln, Dezember 2013

(Nora Peine)